CHEMISTRY OF NATURAL PRODUCTS

FLAVONES & BIFLAVONES

(SUMMARY)

THESIS SUBMITTED FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY IN
CHEMISTRY

September, 1971

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S U M M A R Y

CHEMISTRY OF NATURAL PRODUCTS :

The investigations on biflavonyls from six Gymnosperms are described in this thesis.

The complex mixtures of biflavonyls from the leaf extracts of Araucaria bidwillii, A.cookii, A. cunninghamii, Agathis alba and A. palmerstonii were examined. The presence of agathisflavone series seems to be characteristic of the order.

Biflavonyls from four Gymnosperms were investigated in detail. The leaf extracts on solvent fractionation, column chromatography followed by preparative thin-layer chromatography gave homogeneous components. The structure of each compound has been elucidated by UV, NMR and Mass Spectral studies. Chemotaxonomic significance of biflavonyls is indicated. The biflavonyls obtained from each source is detailed below:-

Araucaria bidwillii

1. 4'-O-Methylamentoflavone (Bilobetin)
2. 7-O-Methylcupressuflavone
3. 7-O-Methylagathisflavone
4. 7,7"-Di-O-methylcupressuflavone (Biegenkwanin)
5. 7,7"-Di-O-methylagathisflavone.

The 7,7"-di-O-methylagathisflavone is a new optically active biflavonyl.

Agathis alba :

1. Agathisflavone
2. 7-O-Methylagathisflavone
3. 7-O-Methylcupressuflavone
4. 4"',7-Di-O-methylagathisflavone
5. 7,7"-Di-O-methylcupressuflavone.

The parent agathisflavone and 7-O-Methylcupressuflavone are being reported for the first time.

Araucaria cookii :

The phenolic extractives of Araucaria cookii yielded ten biflavonyls in pure form belonging to all the known series of biflavonyls. The four major constituents have already been reported. (d) ,

1. 7,7"-Di-O-methylcupressuflavone
2. 4',4"',7"-Tri-O-methylamentoflavone
3. 4',4"',7,7"-Tetra-O-methylamentoflavone
4. 4',4"',7,7"-Tetra-O-methylcupressuflavone.

The following additional constituents have been isolated and characterised, (c)

5. Hinokiflavone
6. 7"-O-Methylamentoflavone
7. 4"',7-Di-O-methylagathisflavone
8. 4',7"-Di-O-methylamentoflavone
9. 4',7,7"-Tri-O-methylcupressuflavone
10. Sciadopitysin.

The minor constituent notet6uflavone has now been revised to 7"-O-Methylamentoflavone. Araucaria cookii constitutes a second source for the occurrence of 4',7"-Di-O-methylamentoflavone and 4',7,7"-tri-O-methylcupressuflavone.

Cephalotaxus drupacea :

1. Sequoiaflavone
2. Ginkgetin
3. Sciadopitysin.

Although it is very difficult to suggest any taxonomic correlation only on the basis of distribution of biflavonyls, but their abundance mainly in Gymnosperms may help to bring about certain generalizations which can be of great help to "Chemotaxonomists".

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A__C__K__N__O__W__L__E__D__G__E__M__E__N__T

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C__O__N__T__E__N__T__S

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THEORETICAL

Among the important plant colouring matters used for dyeing and printing in the middle ages or earlier were weld (Reseda luteola) young fustic (wood of Rhus Colinus); old fustic (wood of Chlorophora tinctoria);quercitron bark (Quercus tinctoria), and Persian berries (from various species of Rhamnus), which gave yellow, orange, brown and olive shades on aluminium, tin, chromium and iron mordants. According to Colour Index (1956) they find considerable use even at present time, especially old fustic, osage orange (from the wood of Maclura pomifera which also contains morin), and quercitron (Flavine) in the U.S.A., for dyeing silk, wood, nylon and leather, for calico printing, and for shading logwood blacks.[1]

The first flavone to be isolated in the pure state was chrysin from poplar buds (Piccard,1864). Latter studies (1879 onwards) on plant colouring matters led to the isolation, structure determination and synthesis of a large number of flavonoids. They vary in oxidation states, the catechin representing the lowest and the flavonols the highest oxidation levels. Many interconversions of the flavonoids are possible in the laboratory as shown in chart.1.

During the last few years there has been a revival of interest in the chemistry of flavonoids. The term flavonoid covers a large group of naturally occurring compounds in which two benzene rings are linked by a propane bridge (C_6-C-C-C-C_6, except in isoflavones in which the arrangement is C_6-C-C(-C_6)-C). The flavonoids include chalcones, dihydrochalcones, aurones, flavanones, flavones, Isoflavon

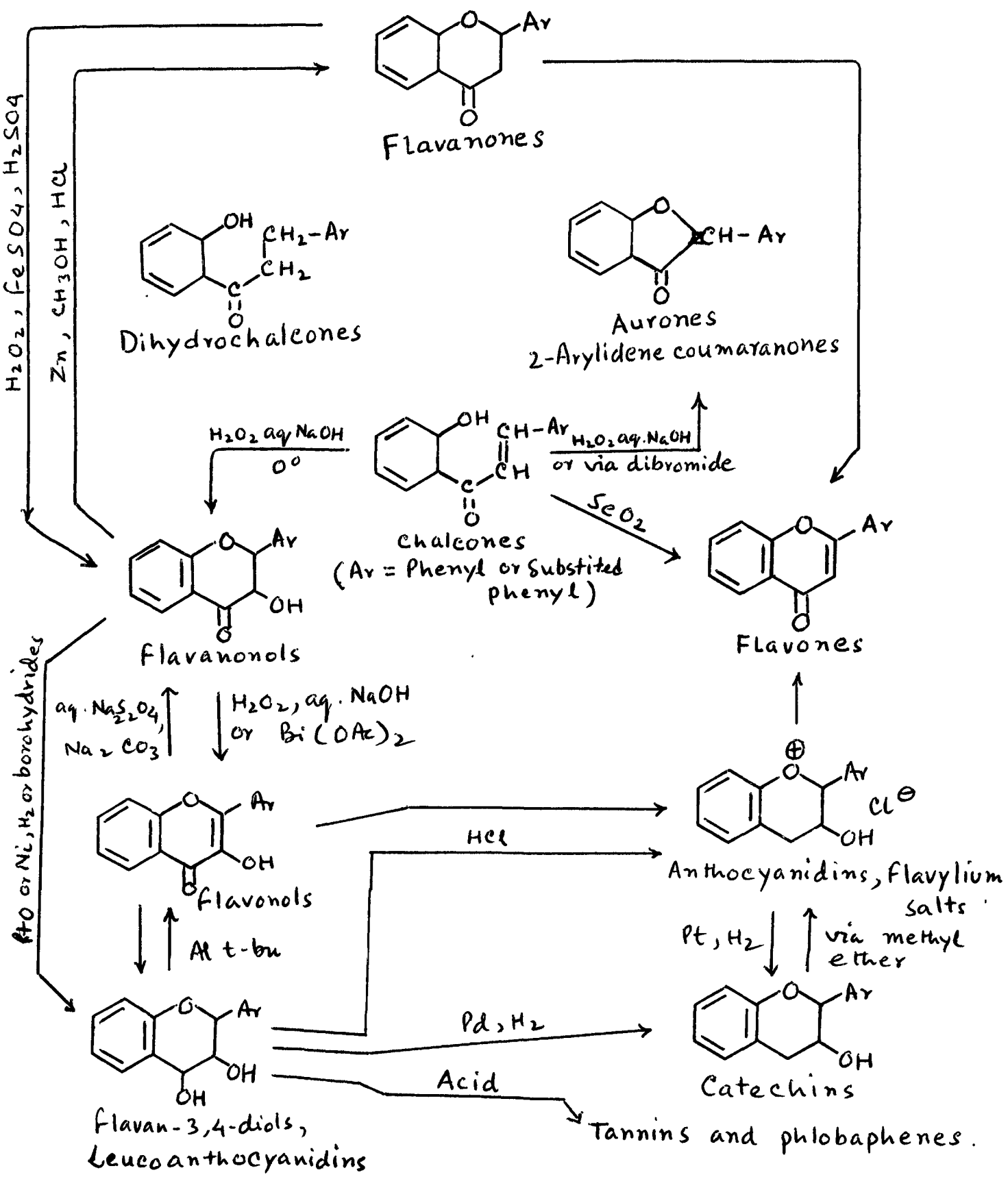

Flavanones
Dihydrochalcones
Aurones
2-Arylidene coumaranones
chalcones
(Ar = Phenyl or Substited phenyl)
Flavanonols
Flavones
Flavonols
Anthocyanidins, Flavylium salts
Catechins
Flavan-3,4-diols, Leucoanthocyanidins
Tannins and phlobaphenes.
H_2O_2, $FeSO_4$, H_2SO_4
Zn, CH_3OH, HCl
H_2O_2 aq NaOH
0°
H_2O_2 aq. NaOH or via dibromide
SeO_2
aq. $Na_2S_2O_4$, Na_2CO_3
H_2O_2, aq. NaOH or $Bi(OAc)_2$
HCl
Al t-bu
Pt, H_2
via methyl ether
Pd, H_2
Acid
Pt or Ni, H_2 or borohydrides

CHART-I

flavonols, 2,3-dihydroflavonols (flavanonols), flavan-3,4-diols (leucoanthocyanidins), anthocyanidins, and catechins.

Numerous physiological activities have been attributed to flavonoids.[2,3] The potent uses of flavonoids may be listed as vitamin P activity (i.e. the property of reducing the capillary fragility and permeability)[4]; diuretic action;[5-7] treatment of allergy; protection against x-rays and other radiation injuries[8]; cure of frostbite;[9-11] antibacterial activity[12]; prophylactic action[13,14]; oestrogenic activity[15-17] and antitumor effects.[18]

The flavonoids are of commercial interest as antioxidants. The antioxidant property of a number of flavonoids has been studied.[19-21] Seshadri et al[22,23] screened twentyseven flavonoids as antioxidants for lard. Robinetin and Gossypetin were claimed as the most potent. The insolubility of hydroxyflavones in fats is a disadvantage in their use as antioxidants. To overcome this a number of C- and O-alkylated flavones have recently been synthesised and tested for their antioxygenic activities.[24,25] 7,8-Dimethoxy-2',3,5'-trihydroxyflavone, 6-ethyl-2',3,5', 7-tetrahydroxyflavone and 6-dodecyl-2', 3,5',7-tetrahydroxyflavone increased the keeping quality of milk powder.[26]

Some years back, a West German drug firm discovered that the extracts of the leaves of maiden hair tree (Ginkgo biloba), have useful medicinal properties and increased blood flow in cerebral and peripheral areas. The detailed studies carried out by Nakazawa[27] and Kawano[28] (in Japan) and Baker[29] (in U.K.) lead to the discovery of biflavonyls- a new class of compounds.

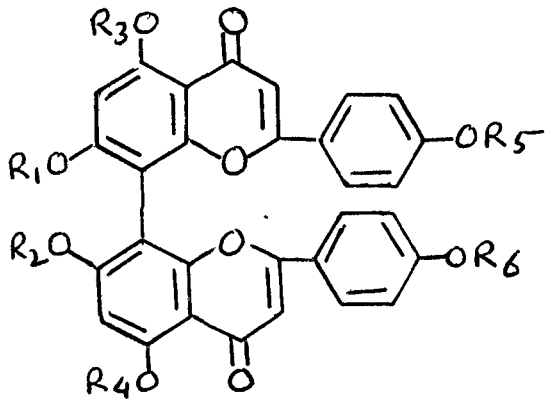
The biflavonyls may be classified into (a) biphenyl type

biflavonyls and (b) biphenyl ether type biflavonyls. The biphenyl types, dependent upon the various modes of interflavonyl linkage and oxidation level may be subdivided into the following classes which are characterised by having two flavonoid C_{15} units.

A. BIPHENYL TYPE BIFLAVONYLS:

1. 4',4"',5,5",7,7"-Hexahydroxy-8,8"-biflavonyl (Cupressuflavone)[30]

This class of biflavonyls is represented by six members. Cupressuflavone is the parent compound while the other five are its partial methyl ethers.[30-36]



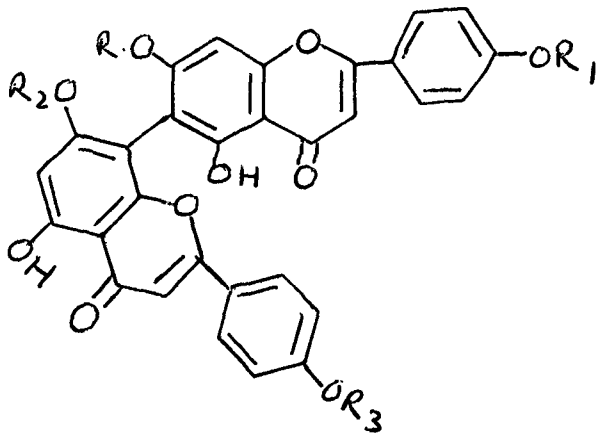
(I)

		R_1	R_2	R_3	R_4	R_5	R_6
(a)	Cupressuflavone[30,31]	H	H	H	H	H	H
(b)	Mono-O-methyl-[32,33]	Me	H	H	H	H	H
(c)	Di-O-methyl-[32-34]	Me	Me	H	H	H	H
(d)	Tri-O-methyl-[35,36]	Me	Me	H	H	Me	H
(e)	Tetra-O-methyl-[34]	Me	Me	H	H	Me	Me
*(f)	Penta-O-methyl-[37]	Me	Me	Me	H	Me	Me

* Synthetic.

2. 4',4"',5,5",7,7"-Hexahydroxy-6,8"-biflavonyl (Agathisflavone):

This class has been recognised very recently and includes only four members.[32,33,38] Agathisflavone, the parent compound, was for the first time obtained during isomerisation of cupressuflavone.[31] It has recently been isolated from the leaves of Agathis alba.[39]

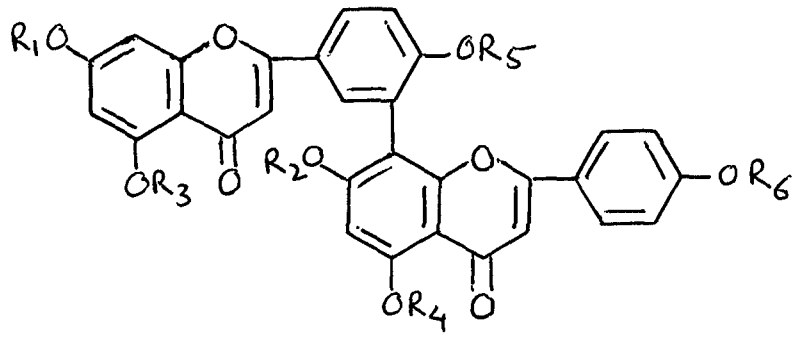


(II)

	R	R_1	R_2	R_3
(a) Agathisflavone[31,39]	H	H	H	H
(b) 7-O-Methyl-(Agathisflavone A)[32,33,38]	Me	H	H	H
(c) 7,7"-Di-O-methyl-[33]	Me	H	Me	H
(d) 4"',7-Di-O-methyl-(Agathisflavone B)[32,38]	Me	H	H	Me

3. 4',4"',5,5",7,7"-Hexahydroxy-3',8"-biflavonyl (Amentoflavone):

This is represented by amentoflavone as the parent compound with twelve of its partial methyl ethers. Kotetsuflavone is now changed to 7"-O-methylamentoflavone.[59,60]

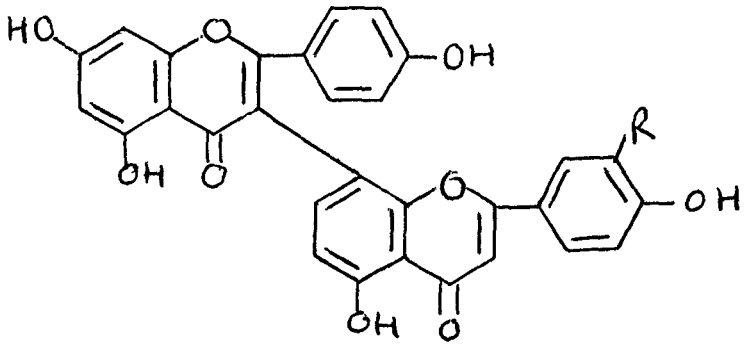


(III)

	R_1	R_2	R_3	R_4	R_5	R_6
(a) Amentoflavone[40-43]	H	H	H	H	H	H
(b) 7-O-Methyl-(Sequoiaflavone)[44-46]	Me	H	H	H	H	H
(c) 4'-O-Methyl-(Bilobetin)[33,46,47]	H	H	H	H	Me	H
(d) 7"-O-Methyl-[41,48,59,60]	H	Me	H	H	H	H
(e) 4"'-O-Methyl-(Podocarpusflavone A)[40,49]	H	H	H	H	H	Me
(f) 4',7-Di-O-methyl-(Ginkgetin)[45,46,50,51]	Me	H	H	H	Me	H
(g) 4',4"'-Di-O-methyl-(Isoginkgetin)[49,51,52]	H	H	H	H	Me	Me
(h) 4"',7-Di-O-methyl-(Podocarpusflavone B)[49]	Me	H	H	H	H	Me
(i) 4',7"-Di-O-methyl-[36]	H	Me	H	H	Me	H
(j) 4',4"',7-Tri-O-methyl-(Sciadopitysin)[45,46,49,53]	Me	H	H	H	Me	Me
(k) 4"',7,7"-Tri-O-methyl-(Heveaflavone)[54]	Me	Me	H	H	H	Me
(l) 4',4"',7"-Tri-O-methyl-(Kayaflavone)[52,55,56]	H	Me	H	H	Me	Me
(m) 4',4"',7,7"-Tetra-O-methyl[34,57,58]	Me	Me	H	H	Me	Me

4. 3,8"-Biflavonyls (Saharanflavone) :

Two new biphenyl type biflavonyls of the series have recently been synthesised[40] by dehydrogenation of BGH-II and BGH-III.

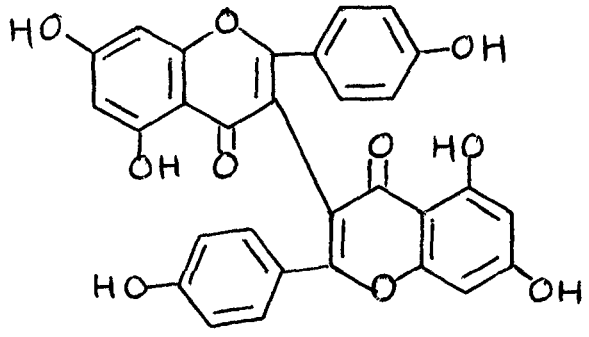


(IV)

		R
(a)	4',4"',5,5",7,7"-Hexahydroxy-3,8"-biflavonyls (WGH-III)[40]	H
(b)	3"',4',4"',5,5",7,7"-Heptahydroxy-3,8"-biflavonyl(WGH-II)[40]	OH

5. 4',4"',5,5",7,7"-Hexahydroxy-3,3"-biflavonyl (V)[61]

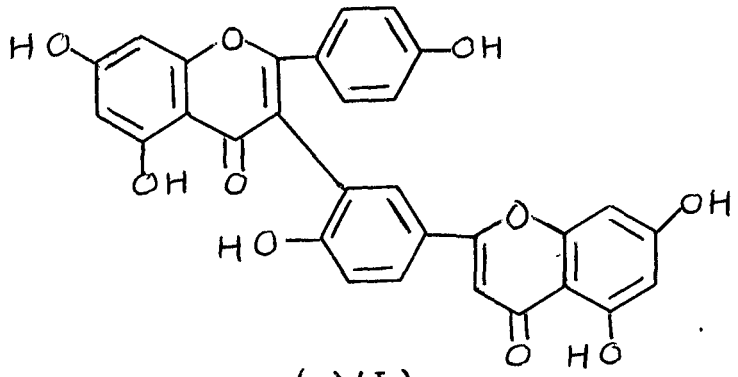
The series comprising of only one member has recently been synthesised by oxidative coupling of apigenin.



(V)

6. 4',4"',5,5",7,7"-Hexahydroxy-3,3"-biflavonyl (VI)[61] :

The sole member of the series has also been obtained during oxidative coupling of apigenin.

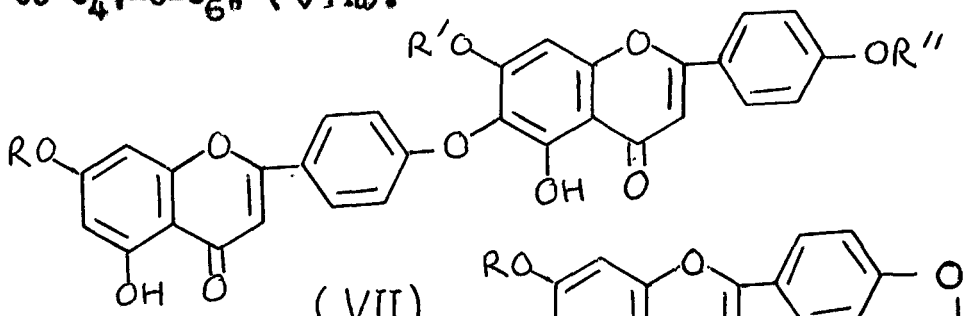


(VI)

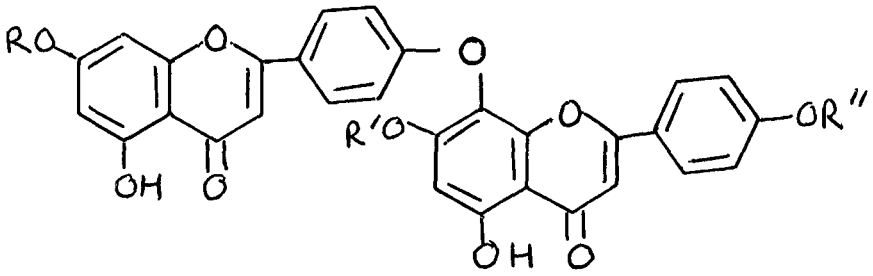
B. BIPHENYL ETHER TYPE BIFLAVONYLS :

4"',5,5",7,7"-Pentahydroxy-4'-O-6"-biflavonyl (Hinokiflavone):

Seven biphonyl ether type biflavonyls have been reported so far. These include the parent hinokiflavone with six others as its partial methyl ethers. They involve C_4'-O-C_6'' linkage (VIIa) between two apigenin units. Earlier hinokiflavone and its derivatives were assigned C_4'-O-C_8'' linkage (VII$_a'$) which has recently been revised to C_4'-O-C_6'' (VIIa).[62]



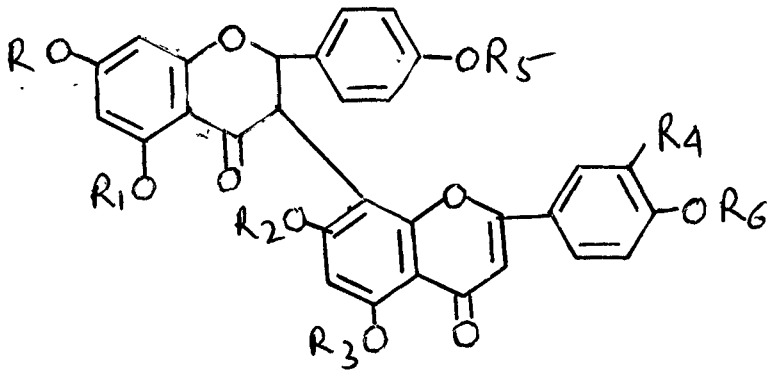
(VII)



(VII')

		R	R'	R"
(a)	Hinokiflavone (4'-O-8")[62]	H	H	H
(a)	Hinokiflavone (4'-O-6")[43,62-64]	H	H	H
(b)	7-O-Methyl-(Neocryptomerin)[49,65]	Me	H	H
(c)	7"-O-Methyl-(Isocryptomerin)[66,67]	H	Me	H
(d)	4"'-O-Methyl-(Cryptomerin - A)[65,68]	H	H	Me
(e)	7,7"-Di-O-methyl-(Chaomocyparin)[65]	Me	Me	H
*(f)	4"',7"-Di-O-methyl-(Cryptomerin-B)[65,68]	H	Me	Me
*(g)	4"',7,7"-Tri-O-methyl-[65,68]	Me	Me	Me

C. FLAVANONE-FLAVONYL TYPE BIFLAVONOIDS(3,8"-flavanone-flavonyl) :



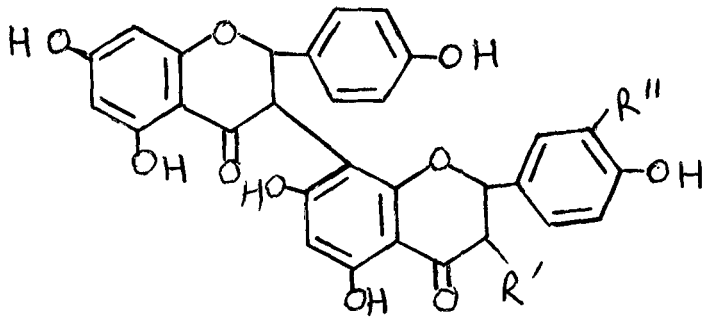
(VIII)

* Synthetic.

	R	R_1	R_2	R_3	R_4	R_5	R_6
(a) BGH-II (Morelloflavone/Fukugetin)[69,70]	H	H	H	H	OH	H	H
(b) 3"'-O-Methyl-[70]	H	H	H	H	OCH_3	H	H
*(c) Hexa-O-methyl-[71]	Me	Me	Me	H	OMe	Me	Me
*(d) Hexa-O-methyl-[71]	Me	H	Me	Me	OMe	Me	Me
*(e) Penta-O-methyl-[71]	Me	H	Me	H	OMe	Me	Me
*(f) Tetra-O-methyl-[72]	Me	H	Me	H	OMe	H	Me
(g) BGH-III (Talbotaflavone/Volkeneiflavone)[39,73,74]	H	H	H	H	H	H	H

D. FLAVANONE-FLAVANONE TYPE BIFLAVONOIDS[75-77] (3,8"-biflavanonyl):

The class (GB series) comprises of reduced heterocyclic systems. Four members are reported to occur in nature. They are derived from naringenin linked with a naringenin or aromadendrin or taxifolin through C_3-$C_{8"}$ linkage.



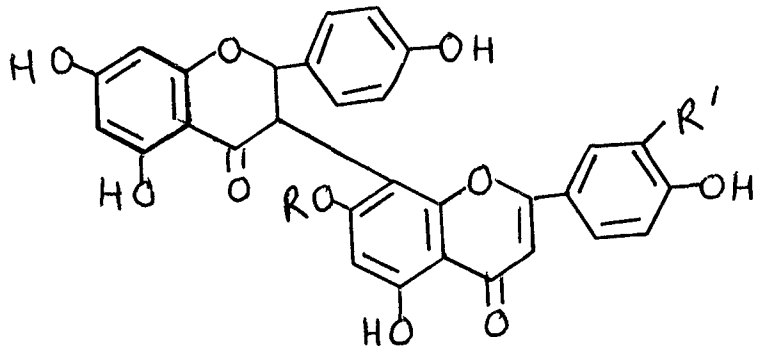
(IX)

	R'	R"
(a) GB-1[75-77]	OH	H
(b) $GB\text{-}1_a$[75-77]	H	H
(c) GB-2[75-77]	H	OH
(d) $GB\text{-}2_a$[75-77]	OH	OH

* Synthetic.

E. BIFLAVONOID GLYCOSIDES :

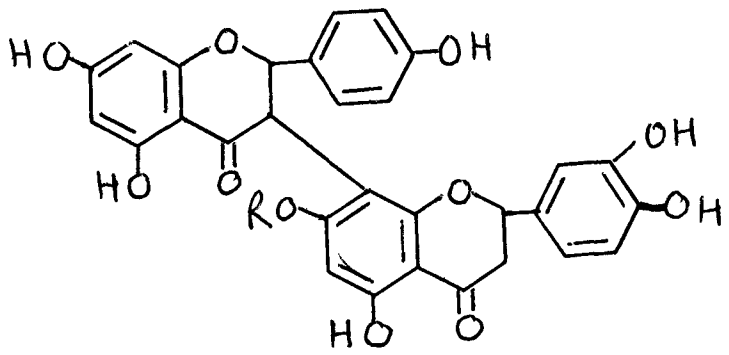
M.Konoshima et al[71,78] have recently isolated fukugiside (Xa) and spicatoside (Xb) from Garcinia spicata and xanthochymuside (XI) from Garcinia xanthochymus.



(X)

(a) Fukugiside[71] R'=OH; R = β-D- gluc.

(b) Spicatoside[78] R'= H; R = "



(XI)

(XI) Xanthochymuside[78] R = β -D-gluc.

F. OPTICALLY ACTIVE BIFLAVONOIDS :

The biflavonoids known till 1968 were found to be optically inactive. Carl Djerassi examined the rotatory dispersion characteristics of the two members of the biphenyl type biflavonyls, isoginkgetin and sciadopitysin in range of 390-700 mμ, but found no optical activity. The argument, however, used for optical inactivity in this series was that all the ortho positions of the biphenyl residue were not substituted. An interesting comment on the optical activity of biphenyl type biflavonyls was made by Seshadri et al.[30] The structure of cupressuflavone incorporates a biphenyl system in which all the ortho positions are substituted by oxygen atoms. However, no optical activity could be detected either in the natural pigment or any of its derivatives.

Recently a large number of naturally occurring optically active biphenyl type biflavonoids have been reported (Table-I). The optical activity has been attributed to the phenomenon of "atropisomerism." However, in fukugetin and xanthochymusside the optical activity may either be due to the asymmetric centre (C_3) alone or to both the asymmetric centre and restricted rotation.

T A B L E - I

OPTICALLY ACTIVE BIFLAVONOIDS

Biflavonoids	$[\alpha]_D^{a,b,c,d,e,}$ (pyridine)	Source
Amentoflavone (IIIa)	+9[a]	Podocarpus gracilior[52]
Cupressuflavone (Ia)	+100[b]	Thuja orientalis[43]
	+63[c]	Cupressus torulosa[59]
7,7"-Di-O-methylcupressuflavone (Ic)	+65[b]	Araucaria cookii[34]
	+37.5	Araucaria cunninghamii[59]
4',4"',7,7"-Tetra-O-methylcupressu-flavone (Ie)	+30[c]	Araucaria cookii[57]
4',4"',7,7"-Tetra-O-methylamento-flavone (IIIm)[34]	+41[c]	" "
Kayaflavone (IIIl)[59]	+18[c]	" "
Fukugetin (VIIIa)	+170[c]	Garcinia spicata[70]
Podocarpusflavone A (IIIe)	-6[a]	Podocarpus gracilior[52]
4"',7-Di-O-methylagathisflavone(IId)	-55[c]	Agathis plamorstonii[38]
7-O-Methylagathisflavone (IIb)	-50[c]	" "
7,7"-Di-O-methylagathisflavone(IIc)	-12.5	Araucaria bidwillii[33]
Xanthochymuside (XI)	-40[e]	Garcinia xanthochymus[78]
7"-O-Methylamentoflavone (IIId)	+18.2	Araucaria cunninghamii[59,60]
4',7"-Di-O-methylamentoflavone (IIIi)	+22.7	" "

a=40°, b=34°, c=29°, d=25° and e=20°

STRUCTURE DETERMINATION OF BIFLAVONOIDS :

The problem of structure determination of biflavonoids is a complex one because of (a) occurrence of more than one biflavonyl in chromatographically homogeneous fractions with the consequent difficulty in their isolation in pure form (b) insolubility in the usual organic solvents and (c) the intricate problem of establishing the interflavonyl linkage.

The various methods generally used for structural determination may be classified as under:-

1. Colour reactions
2. Physical methods
   (a) Chromatography
   (b) UV Spectroscopy
   (c) IR Spectroscopy
   (d) NMR Spectroscopy
   (e) Mass Spectroscopy
3. Degradation
4. Synthesis

1. COLOUR REACTION :

The usual reagents for detecting the presence of flavonoids[79-81] (monomers) in plant materials have been found equally useful in biflavonoids. The only exception is that unlike monomers all kinds of biflavonoids give positive test with zinc and hydrochloric acid, a test characteristic of flavononols.[81]
The observation is under investigation.[59]

2. PHYSICAL METHODS :

The physical methods generally employed in structure elucidation are chromatography, UV, IR, NMR and mass spectroscopy.

(a) CHROMATOGRAPHIC METHODS:

A number of papers and review articles have appeared on the separation and identification of flavonoid pigments, specially by paper chromatography in aqueous and alcoholic solvent systems.[82-85] Recently extensive thin-layer chromatographic studies of biflavonyls, their partially and fully methylated derivatives have been carried out in our laboratories.[86] Benzene-pyridine-formic acid (36:9:5) and benzene-pyridine-ethyl formate-dioxan (5:1:2:2) have been found as the most satisfactory developing solvent systems both for qualitative as well as quantitative purposes. Further, the relative differences in R_f values of the complete methyl ethers (even in traces) coupled with the characteristic fluorescence in UV light (Benzene-pyridine-formic acid) were found to be of some help in their identification.[86,87]

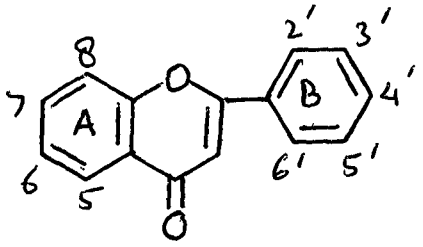
Baker et al[24] and Kawano et al[52] have used counter current distribution between ethyl methyl ketone and a borate or phosphate buffer (definite pH) for the separation of individual biflavonyls from isomeric mixtures as well as from mixtures of biflavonyls of different series.

(b) ULTRAVIOLET ABSORPTION SPECTRA :

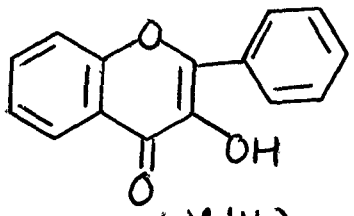
The UV spectra of flavonoids have been thoroughly studied and reviewed by Jurd.[88]

Flavones (XII) and flavonols (XIII) generally exhibit high intensity absorption in the 300-380 mμ region (band I) and the 240-270 mμ region (band II).[89,90] The position and intensity of the

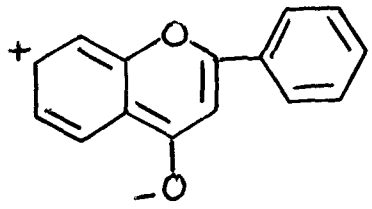
λ max of the absorption bands varies with the relative resonance contributions of the benzoyl (XIV), cinnamoyl (XV) and pyrone ring (XVI) groupings to the total resonance of the flavone molecule.



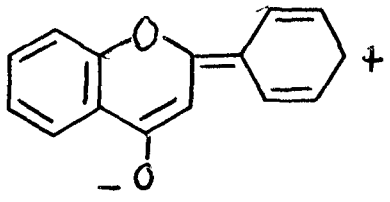
(XII)



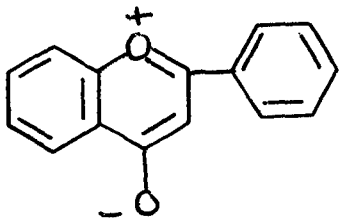
(XIII)



(XIV)



(XV)

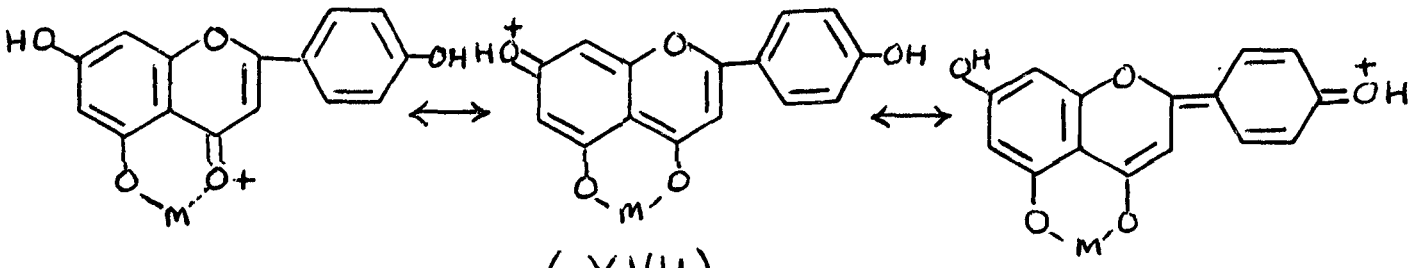


(XVI)

Although these groupings interact, the spectra of substituted flavones and flavonols in the neutral and alkaline solutions suggest that band I is associated chiefly with absorption in the cinnamoyl grouping (XV) and band II with absorption in the benzoyl grouping (XIV).[91]

Spectra of Aluminium Complexes-Location of 5 and 3-hydroxyl groups:

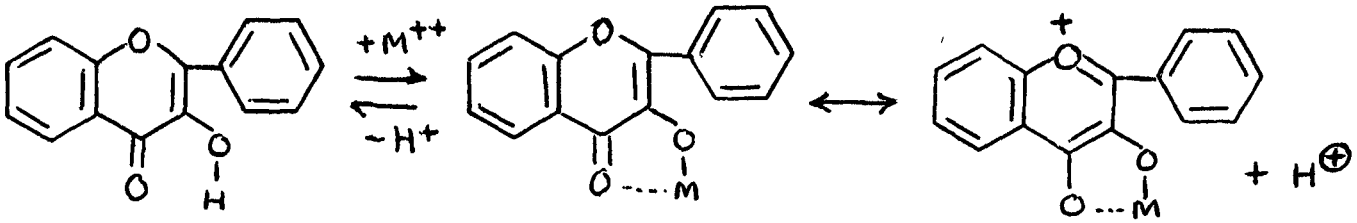
5-Hydroxyflavones and 5-hydroxy flavonols in which the 3-hydroxyl group is protected, form stable yellow complexes of the type (XVII)[92], which result in the considerable bathochromic shifts



(XVII)

of bands I and II. In flavone this shift is of the order of 20-45 mμ.

3-Hydroxyflavones readily form aluminium complexes which are stable even in presence of dilute hydrochloric acid. As a result of complex formation, flavonols produce a flavylium structure (XVIII) which is greatly stabilized by its quasi-aromatic character.



(XVIII)

The bathochromic shift of the flavonol band I to the complex band I is consistently in the order of 60 mμ. A shift of this magnitude is, therefore, reliable evidence for the presence of a free 3-hydroxyl group.

Spectra in Alcoholic Sodium Acetate-Location of 7-Hydroxyl Group :

Sodium acetate is sufficiently basic to ionize hydroxyl groups located at positions 7,3 and 4' of the flavone nucleus. Hydroxyls at other positions are unaffected. Ionization of 3- and 4'- hydroxyls produces bathochromic shifts of band I, but does not affect the position of band II. Since band II is associated mainly with absorption in A ring, ionization of a 7-OH group results in a pronounced bathochromic shift of this band. Flavones and flavonols which contain a free 7-hydroxyl group may, therefore, be detected by the 8-20mμ bathochromic shift of the low wave length band on the addition of a little fused sodium acetate.[91]

Detection of a 3,4'-Dihydroxyl Grouping in Flavonols :

Jurd and Horowitz[91] found that flavonols in which the hydroxyl group at either C_3 or C_4, is protected by methylation or glycosidation are stable in sodium ethoxide and that their stability is not appreciably influenced by other hydroxyl groups in the molecule. These compounds show normal spectral shifts i.e. the long wavelength band shifts from 340-380mμ in ethanol to about 380-420mμ in sodium ethoxide.

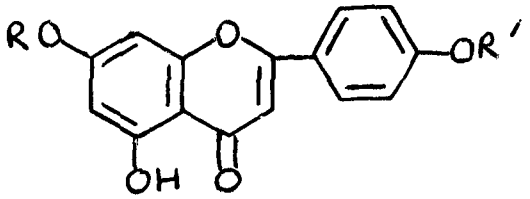
Detection of O-Dihydroxyl Groups :

Boric acid, in the presence of sodium acetate forms chelates with phenolic compounds containing O-dihydroxyl groups. Thus the λ max of band I in luteolin undergoes a bathochromic shift of 15-30mμ on addition of a mixture of boric acid and sodium acetate.[93] The spectra of compounds which do not contain an O-dihydroxyl groups are not appreciably affected.

UV Spectra of Biflavonyls :

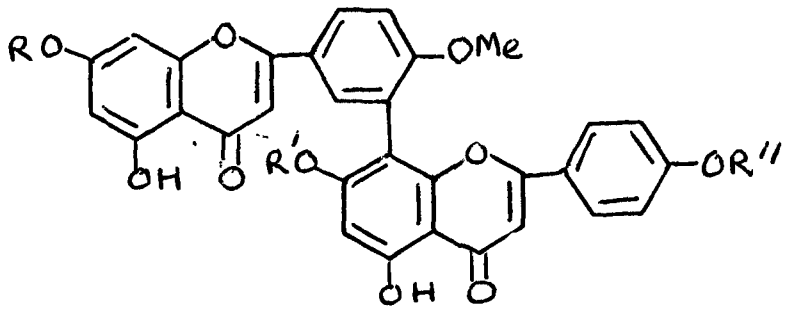
Ultraviolet spectroscopy has been found extremel structure elucidation of biflavonyls.

That the ginkgetin and isoginkgetin are derived fr trihydroxyflavone is apparent by comparison of UV spectr getin, isoginkgetin and their tetraacetates and tetrameth with those of apigenin (XIX), acacetin (XX) and genkwanin and their derivatives.



	R	R'
(XIX)	H	H
(XX)	H	Me
(XXI)	Me	H

Baker et al[29] proposed structure (IIIf) or (IIIg) for ginkgeti on the basis of spectroscopic evidence and degradation products.



(IIIf) R = Me; R'=R"=H

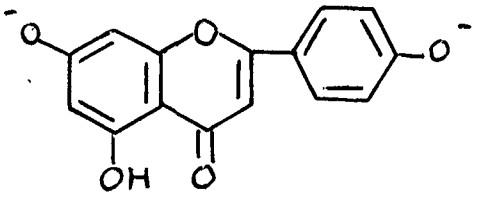
(IIIg) R = R'=H; R"=Me

The study of effect of base upon the ultraviolet spectra of the biflavonyl allowed a decision to be made between the two structures (IIIf) and (IIIg). This effect has been discussed in detail for isoginkgetin (IIIg) and sciadopitysin (IIIi) as well.[29]

The positions of maximal absorption of biphenyl type biflavonyls and their derivatives are very similar to apigenin and its derivatives, the molecular extinction coefficient of biflavonyls are approximately double as compared to the corresponding monomers. This shows the presence of two isolated chromophores of flavonoids per molecule of biflavonyl.

(c) Infrared Spectroscopy[29,94-97]

The infrared spectra[29] of 5-hydroxy biflavonyls shows strong bands at 1660 cm^{-1} as do those of mono-5-hydroxy flavonoids. The band is characteristic of 5-hydroxyflavones (XXII) and although this hydroxyl group is internally hydrogen-bonded the effect of 5-O-alkylation and 5-O-acylation is opposite to that shown in the case of simple O-hydroxyketones. Because of internal hydrogen-bonding in O-hydroxyketones, the carbonyl bands of these compounds show a shift to higher frequencies on either O-alkylation or O-acylation. However, a similar comparison of the infrared spectra of 5-hydroxyflavones and 5-hydroxychromones with the spectra of their 5-O-alkyl, and 5-O-acyl derivatives shows a shift in the opposite direction, that is to lower frequencies. In practice this effect is very useful in diagnosing the presence of a 5-hydroxyflavone structure.



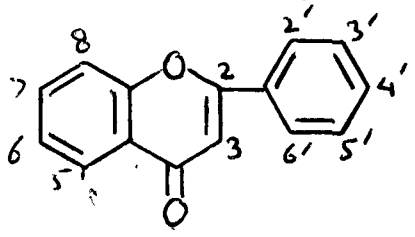
(XXII)

(d) NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY :

The application of NMR spectroscopy has proved to be the most powerful tool in structure determination of flavonoids. The recent technique of preparing silyl derivatives[98,99] for NMR studies has not only overcome the solubility problem but also has contributed towards the simplification of spectra.

The valuable contributions in the field have been made by Batterham and Highet;[100] Mabry;[101] Massicot;[102] Clark Lewis[103] and Kawano.[65]

The most commonly occurring hydroxylation pattern in natural flavonoids is 4',5,7-trihydroxy system. The chemical shifts of the protons of ring A and B prove to be independent of each other but are affected by the nature of ring C.



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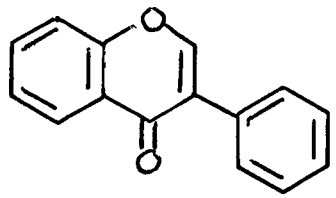
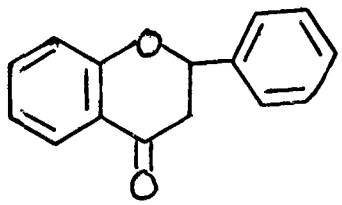


(XXIII)



(XXIV)

The two A-ring protons of flavonoids with the 5,7-hydroxylation pattern give rise to two doublet (J_{meta}= 2.5 cps) between τ3.3-4.0 from tetramethyl silane. There are, however, small but predictable variations in the chemical shifts of the C-6 and C-8 proton signals depending on the 5- and 7-substituents. In flavanones the 6,8-protons give a single peak near τ4.05; with the addition of a 3-hydroxy group (flavanonols) the chemical shifts of those protons are slightly altered and the pattern changes to a very strongly coupled pair of doublets. The presence of the double bond in ring C of flavones and flavonols causes a marked downfield shift of these peaks, again producing the two doublet pattern. Out of 6- and 8-protons, the latter appears downfield.

All B-ring protons appear around τ2.3-3.3, a region separate from the usual A-ring protons. The signals from the aromatic protons of an unsubstituted B ring in a flavanone appear as a broad peak centred at about τ2.55. In flavones, the presence of C ring double bond causes a shift of the 2',6' protons and the spectrum shows two broad peaks, one centred at τ2.00 (2',6') and the other at τ2.4 (3',4',5').[100]

With the introduction of a 4'-hydroxyl group, the B ring protons appear effectively as a four peak pattern. This is called A_2B_2 pattern. Introduction of one more substituent to ring B gives the normal ABC pattern.[100] The hydroxy group increases the shielding on the adjacent 3',5' protons and their peaks move substantially upfield. The 2',6' protons of flavanones give signals centred at about τ2.65. Introduction of the 2,3 double bond (flavones and

flavonols) again causes these protons to resonate at much lower-field. Considerable variations are generally found for the chemical shifts of the C-ring protons among the several flavonoid classes. For example, the C-3 proton in flavones (XII) give a sharp singlet near τ3.7. The C-2 proton of isoflavones (XXIII) is normally observed at about τ2.3, while the C-2 proton in flavanones (XXIV) is split by the C-3 protons into a quartet (J_{cis}= 5 cps, J_{trans}= 11 cps) and occurs near τ4.8. The two C-3 protons occur as two quartets ($J_{H-3a,H-3b}$= 17 cps) near τ7.3. However, they often appear as two doublets since two signals of each quartet are of low intensity. The C-2 proton in dihydroflavonols appear near τ5.1 as a doublet (J=11 cps) coupled to the C-3 proton which comes at about τ5.8.[102]

The relative stereochemistry of 3-substituted flavanones can usually be established from a consideration of vicinal coupling constants and the "KARPLUS equation". In all cases, the heterocyclic ring appears to adopt the chair or half chair conformation in which 2-aryl substituent is quasi-equatorial. Massicot & Marthe,[102] analysing the ABX spectrum of heterocyclic ring protons of 6,7-dimethoxyflavanone, have shown the two vicinal coupling constants to be 13.5 and 3.2 cps. The former is clearly a diaxial interaction, thus establishing the equatorial character of 2-aryl group in flavanones. All 3-hydroxy and 3-acetoxy flavanones, which have been examined, exhibit vicinal coupling constants 12 cps and were therefore assigned the trans (diequatorial) configuration,

although in the case of naturally occurring compounds the possibility of epimerization can not be excluded.

The proton of a 5-hydroxyl group next to a C-4 carbonyl of a flavonoid gives rise to a sharp signal at a very lowfield consistent with the strong hydrogen bonding between the two groups.

Methylation of a hydroxyl group commonly produces an upfield shift (~0.2 ppm) of the signals of ortho protons with a somewhat smaller effect on those of para protons and little or no effect on the meta protons. Acetylation of the hydroxyl group, as expected, causes downfield shift of the ring protons.

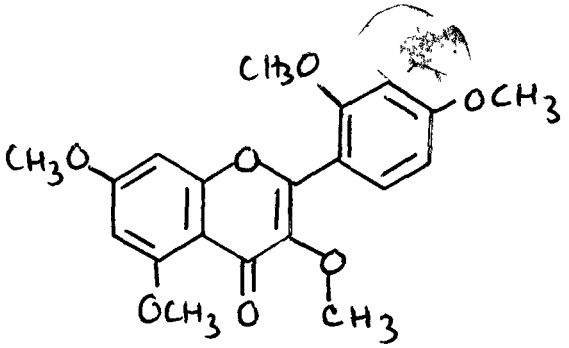
The n.m.r. studies of the majority of workers in biflavonyls, both past and present were restricted to the chemical shifts of acetoxy and methoxy protons leaving each and individual proton unassigned and the vexing problem of interflavonyl[30,75a,105-107] linkage uncertain. The recent use of double irradiation techniques and solvent dependent methoxy shifts[31,34,38,39,56,74,107,108] has contributed significantly in unravelling the fine details of the complex molecules. Due to lack of standard samples of biflavonyls and therefore the spectral data, it was customary to elucidate the structure of a new member with the help of the spectral data of the corresponding monomer. It has been observed that such a choice is compelling but by no means infallible.[34,109-111]

The observations and interpretations of spin-spin splitting are the means by which the sequence of groups in molecules is established by NMR. However, the process of establishing sequence

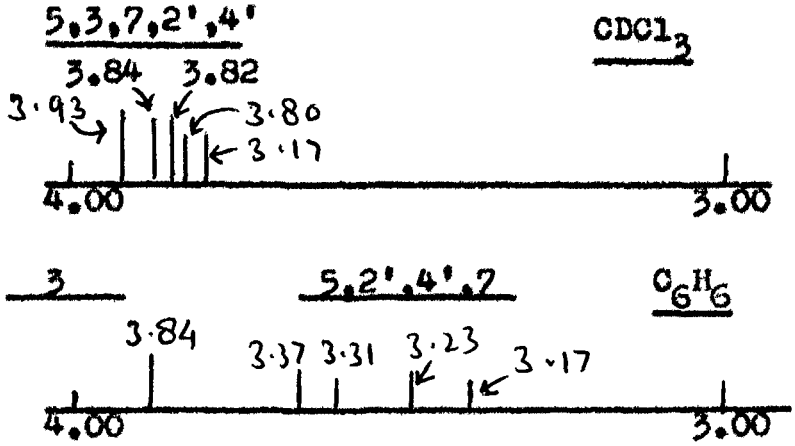
of groups in molecules even on high resolution NMR frequently fails, because, while it may be possible to observe a discrete multiplet from one group of protons it may be impossible to recognise the absorptions of protons to which this group is coupled, since they may be obscured by absorptions of other protons in the molecule. An anciallary technique known as spin decoupling (double resonance) often helps to overcome this difficulty. By the help of double irradiation technique it has been possible to assign each and every proton in biflavonyls.[111]

Recently the solvent dependence of methoxy resonances induced by benzene (relative to comparatively inert solvent, such as CCl_4 or $CDCl_3$) upon electronic, steric and conformational factors has been noted.[112-114] The position and relative orientation of methoxy groups in methoxyflavones can be inferred from benzene induced solvent shifts of methoxy resonances.[108]

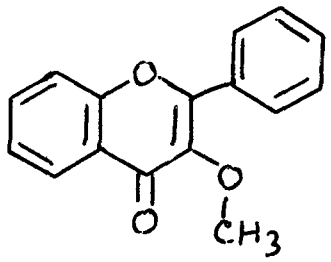
It has been observed by Dudly H. Williams and co-workers[108] that methoxy groups at C-5, C-7, C-4' and C-2' exhibit large positive Δ values (Δ = δ $CDCl_3$ − δ C_6H_6 ≃ 0.5 to 0.8 ppm) in the absence of methoxyl or hydroxyl substituents ortho to these groups (e.g. XXV). This means that aforesaid methoxy signals move upfield



(XXV)

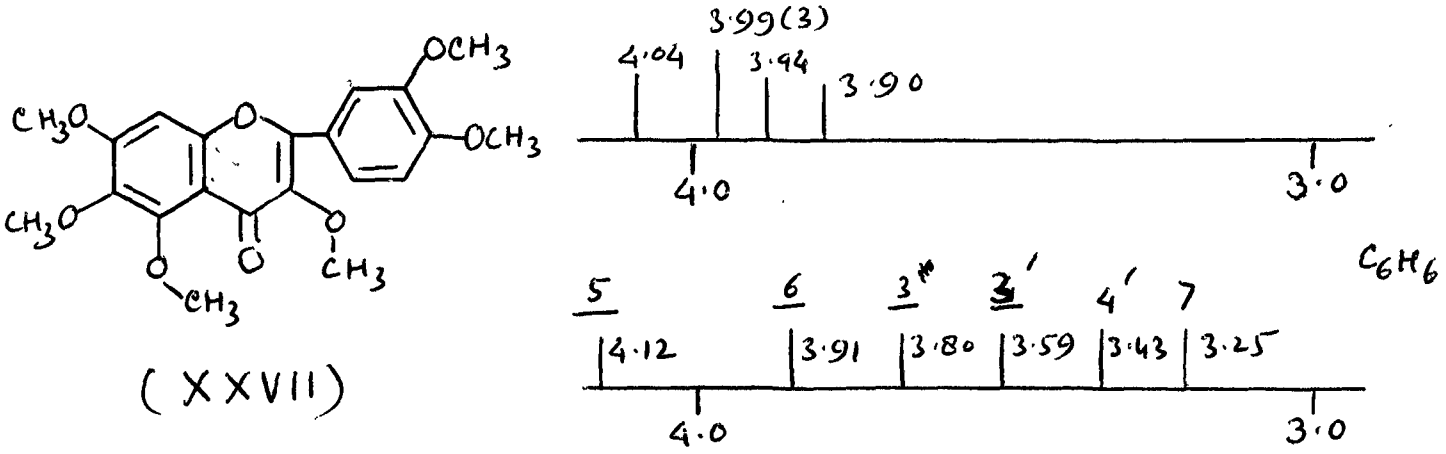


in benzene relative to deuterochloroform. The observation is consistent with the formal ability of all these methoxy groups to conjugate with the electron withdrawing carbonyl group. This conjugation may lead to a decrease in electron density at oxygen atoms of OMe groups in question and so enhance an association with benzene at these electron-deficient sites with the resultant increased shielding effect.[112-114] The C-3 methoxy resonances are in contrast deshielded or only slightly shielded in benzene (Δ = -0.07 to +0.34). This observation strongly suggests that the C-3 methoxy group in general prefers conformation indicated in (XXVI). In this conformation, phase independent association of benzene with the carbonyl group will have deshielding influence on the C-3 methoxy group.[115-117]



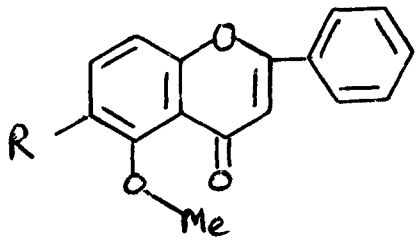
(XXVI)

The solvent shift of an OMe group at C-5 suffers a drastic change in magnitude from a relatively large positive value to a small or negative value in the presence of an OMe at C-6,[(XXVII)] Such a change is in accord with expectations, since the introduction of an ortho OMe group generally causes an algebraic decrease in Δ, and



(XXVII)

in addition a C-6 substituent should lead to a higher population of the conformer in which the Me of the C-5 functionality lies in close proximity to the negative end of the carbonyl dipole (XXVIII) (which is a region of strong deshielding due to benzene association at the carbonyl group).

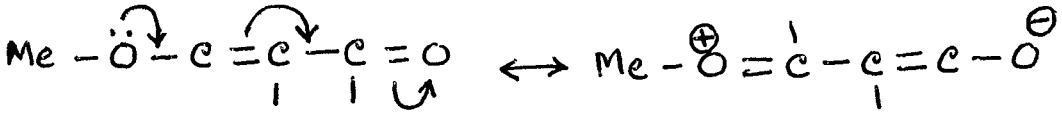


(XXVIII)

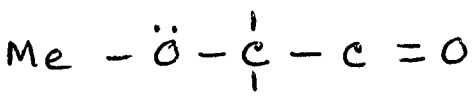
R=OMeor Monoflavonoid

The benzene induced solvent shifts Δ (chloroform/benzene) of certain methoxy groups in flavones are appreciably enhanced by the addition of small quantity (5% v/v) of trifluoroacetic acid (TFA)

to the solution of the flavone in benzene;[118,119] apparently protonation of certain groups enhances the benzene association at these sites. This technique should help to distinguish between methoxy groups which can conjugate with the carbonyl group (XXIX) and those which cannot conjugate in the ground state (XXX).



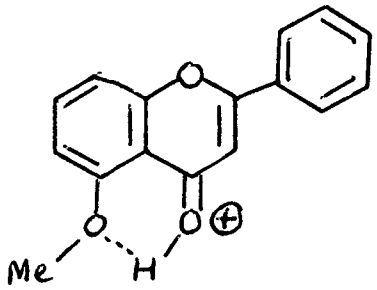
(XXIX)



(XXX)

The basicity of methoxy-groups of type (XXX) is greater than of those of type (XXIX), so the former will be expected to give more positive value of the TFA-addition shift Δ (C_6H_6/C_6H_6-TFA).

In contrast, the TFA-induced solvent-shift Δ ($CDCl_3$/TFA) of a 5-methoxy group has a relatively large negative value (-0.36 to -0.44 ppm), which distinguishes it from the methoxy groups at other sites. A possible explanation for the large TFA-induced solvent shift is the formation of a hydrogen bond between the protonated carbonyl group and the oxygen atom of the 5-methoxy-group (XXXI).[108]



(XXXI)

The carbonyl group will be protonated to a much larger extent in TFA relative to a solution in benzene containing only 5% TFA.

If a 5-methoxy-group is flanked by a substituent like a methoxy group at C-6, its TFA-induced solvent shift is even greater than in the absence of such a group (-0.48 to -0.62 ppm). This characteristic solvent shift should prove very useful in structure elucidation.[119]

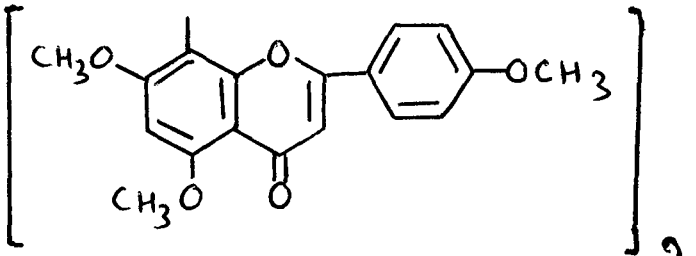
NMR STUDIES ON BIFLAVONYLS:

SOLVENT SHIFT STUDIES OF METHOXY RESONANCES:

The question of implication of either C-8 or C-6 in interflavonyl linkage used to be settled by the relative ease of methylation of the hydroxyl group at position 5.[75a,104] The decision was based upon steric considerations. Later studies revealed that the observation was not of general applicability and had led to erroneous assignments. This intricate problem of interflavonyl linkage both in biphenyl and biphenyl ether type biflavonyls has successfully been solved by benzene induced shift

studies of methoxy resonances.[34,38,40,57,79,107,108] It has been shown that in the fully alkylated compounds, signals of methoxy groups ortho to an aromatic-hydrogen atom move upfield by more than 30 c/s on change of solvent from chloroform to benzene but signals of methoxy groups lacking such a proton move by 0-20 c/s.[120] Downfield shifts in the latter case have also been observed.[108]

Cupressuflavone hexamethyl ether (XXXII) :



(XXXII) $_2$

It is expected from the above discussion that if we have 8-8" linked biflavonyls, both the 5-methoxy groups which are clear and distinct from the rest, below τ6.00 (τ5.88), will along with OMe-7 and OMe-4' move up 50-60 c/s, if, however, the biflavonyl is 6-6" linked, whereas the OMe-7 and OMe-4' will move to the same extent (50-60 c/s), the OMe-5 will shift to a very small or negative value. The results of the solvent induced shift experiment on cupressuflavone hexamethyl ether are shown below (Table-II).

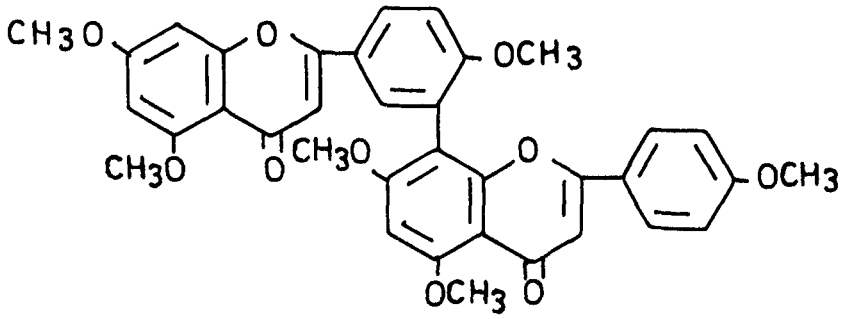
T A B L E -II

BENZENE INDUCED SHIFTS OF METHOXY RESONANCES IN CUPRESSUFLAVONE HEXAMETHYL ETHER

Signals in $CDCl_3$ c/s	Signals in C_6H_6 c/s	Shifts c/s
412	356	+56
386	329	+59
377	302	+75

Thus all the methoxy signals shifted upfield as expected for a C8-C8" linkage.

Amentoflavone hexamethyl ether[57](XXXIII) :



(XXXIII)

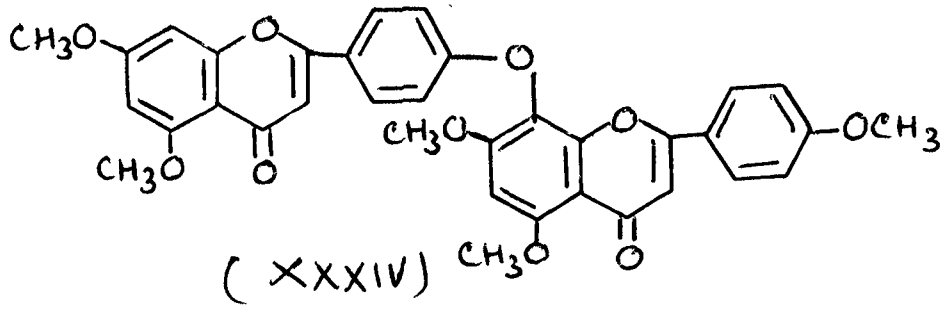
All methoxy groups were shifted upfield on change of solvent from deuterochloroform to benzene as with cupressuflavone hexamethyl ether, showing that every methoxy group has at least one ortho proton and therefore a C-8 rather than a C-6 linkage is indicated (Table-III).

T A B L E -III

BENZENE INDUCED SHIFTS OF METHOXY RESONANCES IN AMENTOFLAVONE HEXAMETHYL ETHER

Signals in 100%$CDCl_3$ 0% Φ D	Signals in 19.5%$CDCl_3$ 80.5% Φ D	Δ c/s
(5"OMe) 5.93	6.35	42
6.06	6.50	44
6.10	6.72	62
6.16	6.75	59
6.23	6.72	49
6.25	6.81	56

Hinokiflavone pentamethyl ether[62] (XXXIV) (Synthetic, C_4'-O-C_8'')



(XXXIV)

The C_6D_6 induced shifts for the methoxy resonances are represented in Fig.I. They are precisely as expected from a compound with five methoxy groups unhindered to solvation by C_6D_6

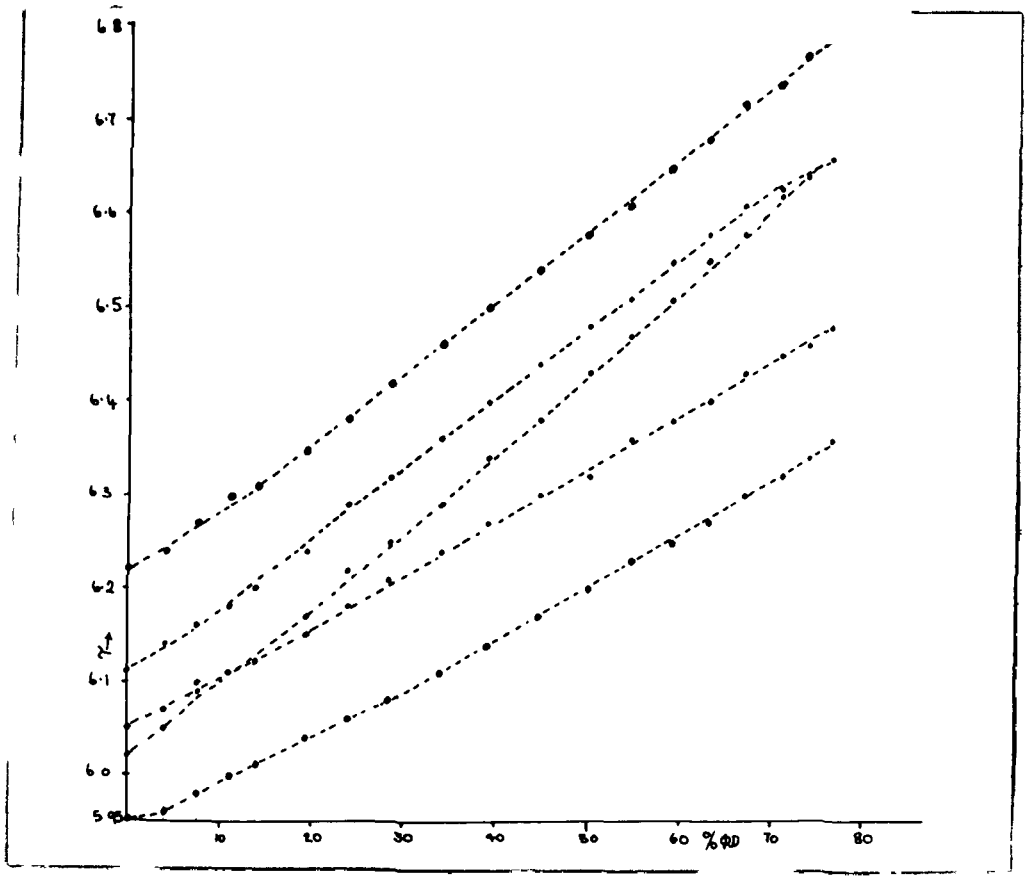
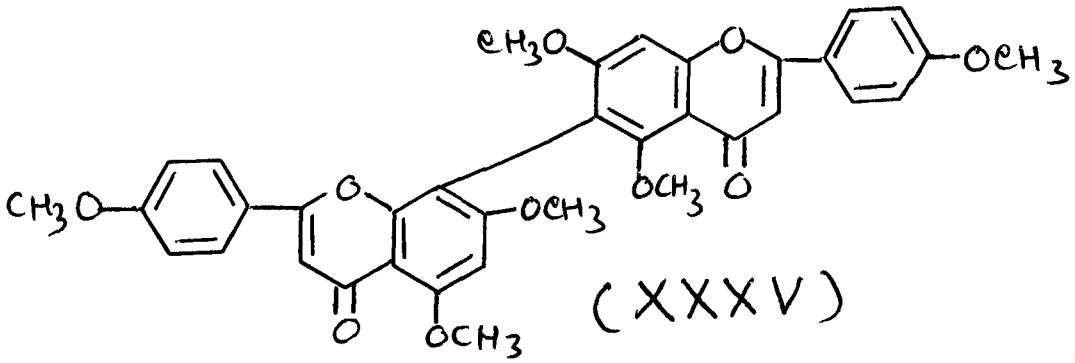


Fig. I

Agathisflavone hexamethyl ether[38] (XXXV) :



(XXXV)

Five methoxy groups showed large upfield shifts (Table-IV, Fig.II). One group was unique in that upto 50% dilution with benzene no shift was seen and then a strong downfield shift was evidenced. It was reasonable to assume that the methoxy group in question was the unique one at C-5, flanked by ring D on one side and a carbonyl group on the other.

T A B L E - IV

BENZENE INDUCED SHIFT OF METHOXY RESONANCES IN AGATHISFLAVONE HEXAMETHYL ETHER

Signals in $CDCl_3$ c/s	Signals in C_6H_6 c/s	Shifts c/s
485	358	+47
390	330	+60
389	335	+54
380	326	+54
375	305	+70
362	385	-23

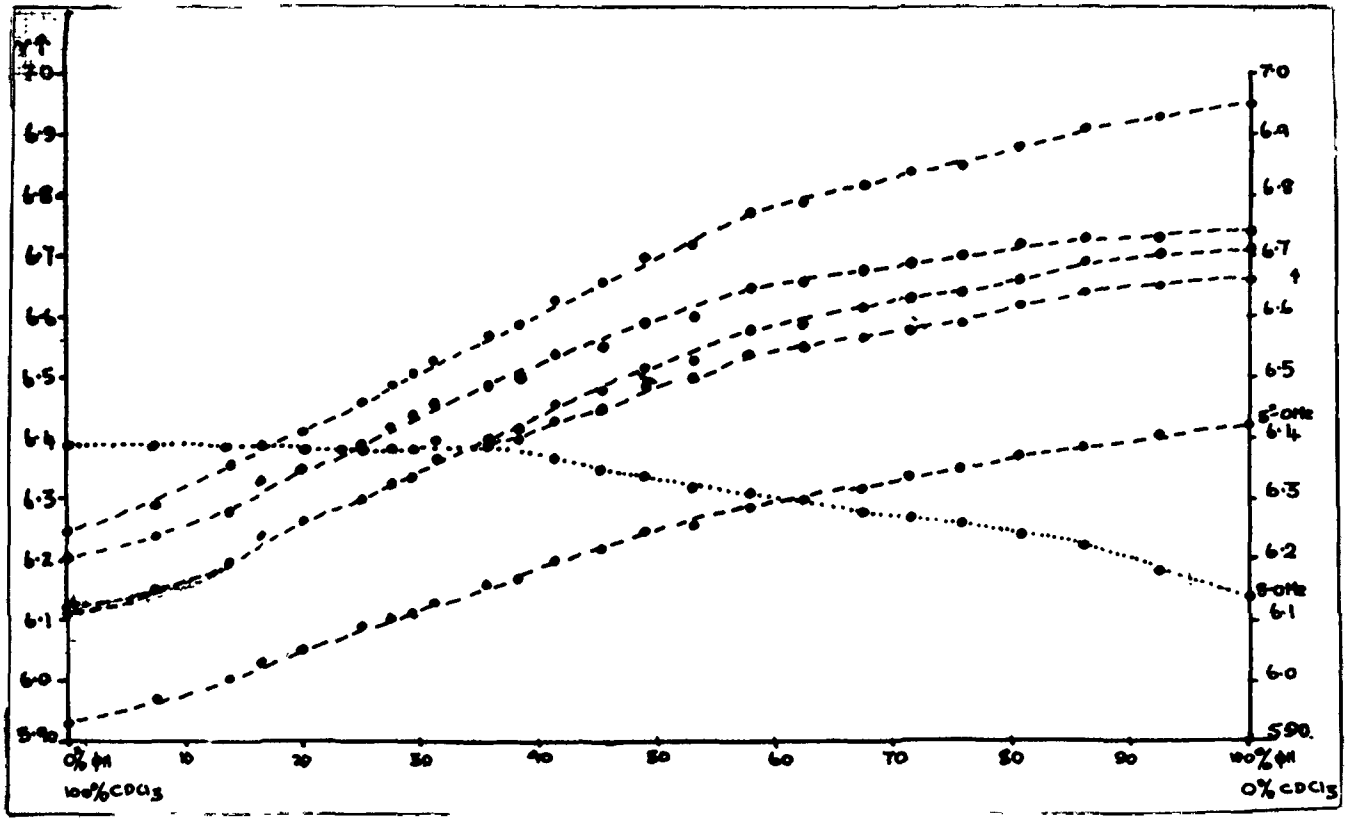
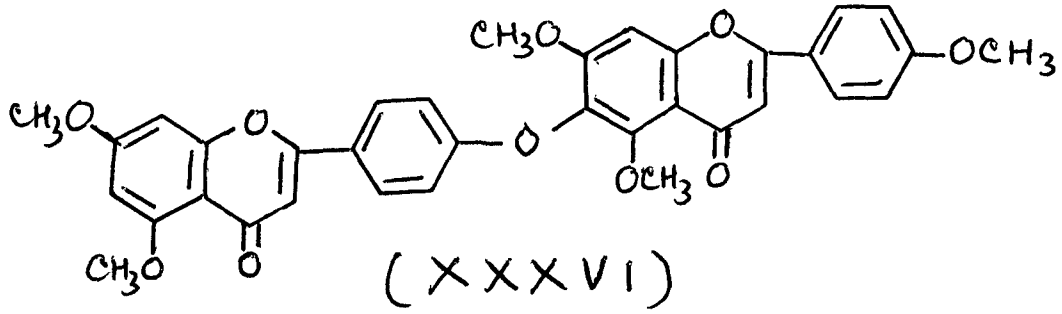


Fig. II

Hinokiflavone pentamethyl ether[62] (XXXVI) ($C_{4'}$-O-$C_{6''}$) :



(XXXVI)

This gave the methoxy shifts as shown in Fig.III. The shifts from 0% C_6D_6 to 80% C_6D_6/20% $CDCl_3$ were 34 c/s, 54 c/s, 57 c/s, 64 c/s and 1 c/s. These shifts are well within the range for four unhindered and one hindered methoxy groups and show that the sample has the structure of $C_{4'}$-O-$C_{6''}$ linked hinokiflavone.

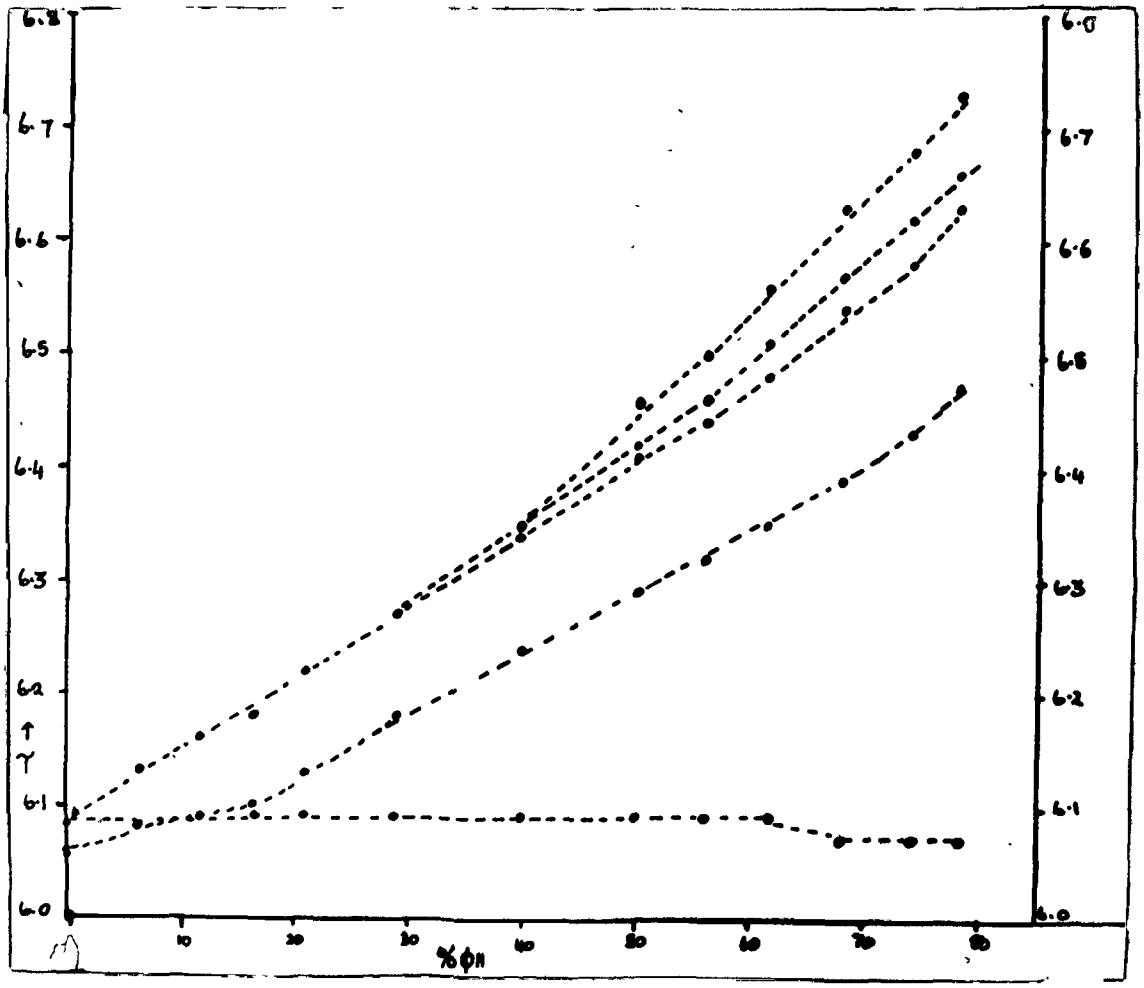
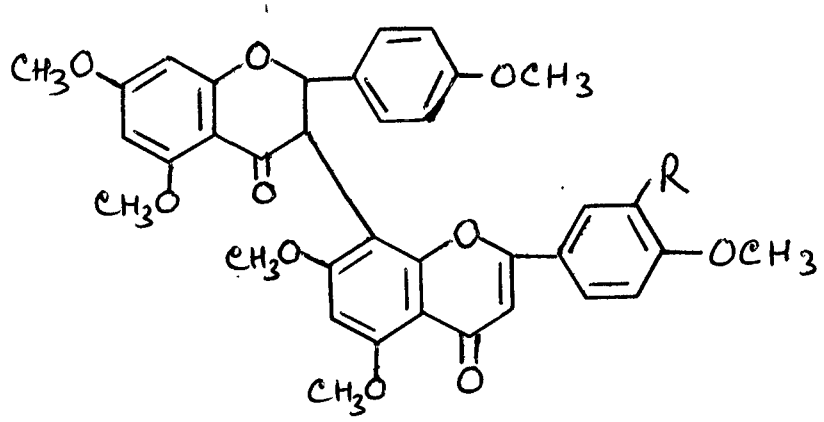


Fig III

BGH-III and II methyl ethers[40,74] (XXXVII & XXXVIII) :

Solvent induced shift studies of methoxy resonances were found useful in BGH series. All methoxy signal (τ6.03-6.36) moved upfield (τ6.5-6.8) Fig.IV and V. This clearly fixed the flavanone substituent at C-8 rather than at C-6 of the flavone unit.



(XXXVII) BGH-III methyl ether, R = H

(XXXVIII) BGH-II methyl ether, R = OMe

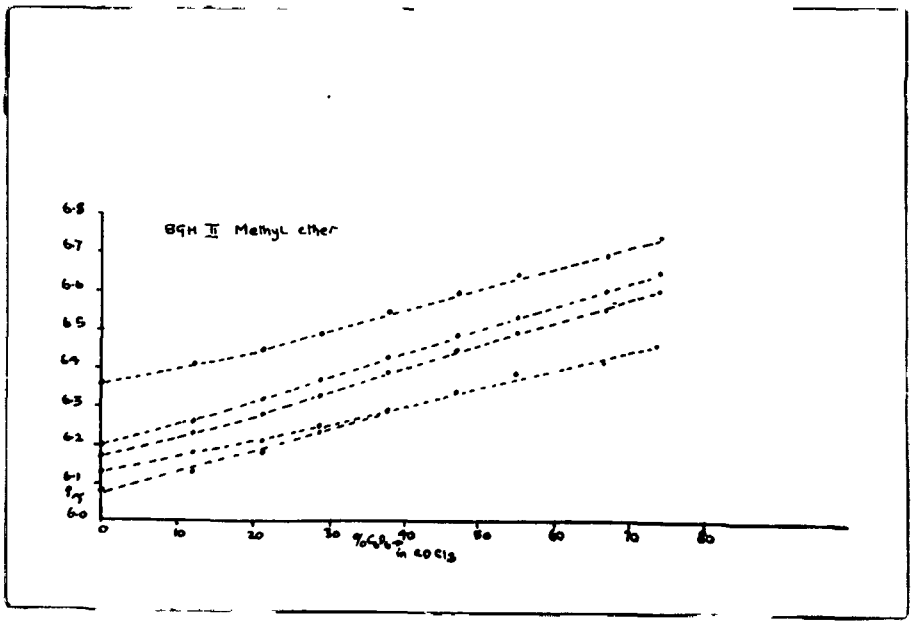

BQH II Methyl ether
6.8
6.7
6.6
6.5
6.4
6.3
6.2
6.1
6.0
δ
0
10
20
30
40
50
60
70
80

Fig. IV

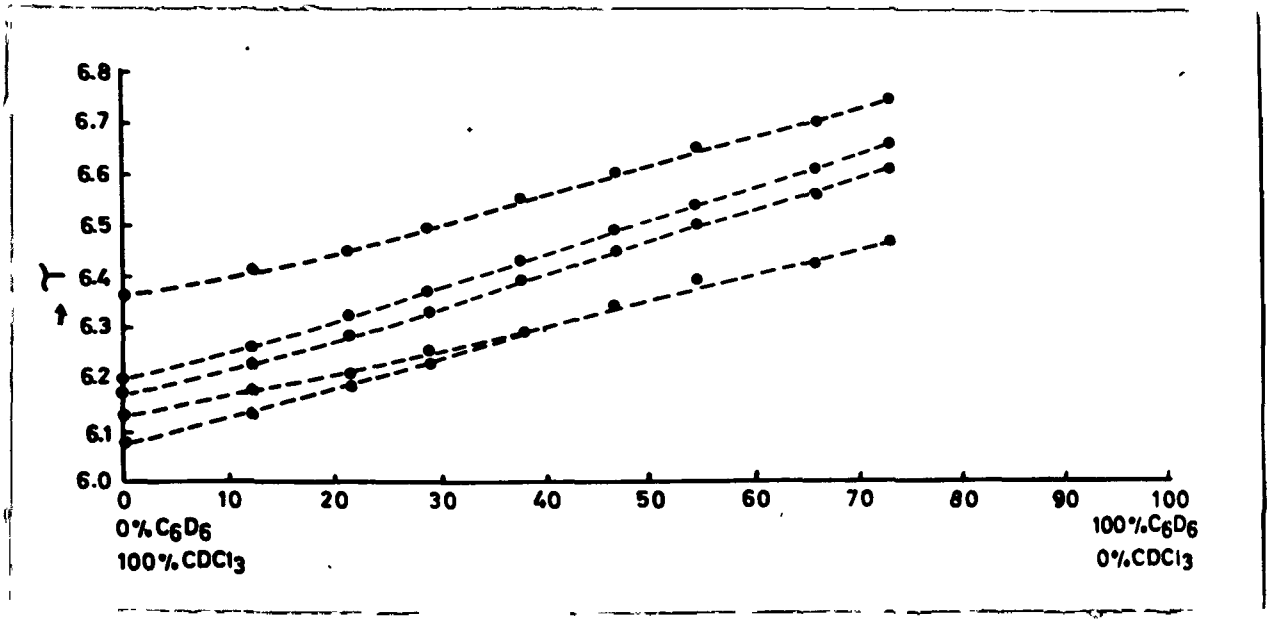
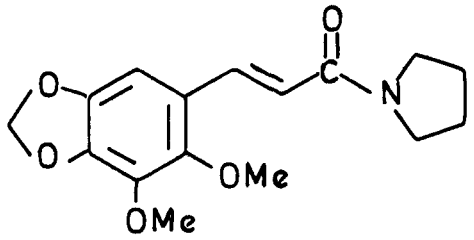

6.8
6.7
6.6
6.5
δ
6.4
6.3
6.2
6.1
6.0
0
10
20
30
40
50
60
70
80
90
100
0% C6D6
100% CDCl3
100% C6D6
0% CDCl3

Fig V

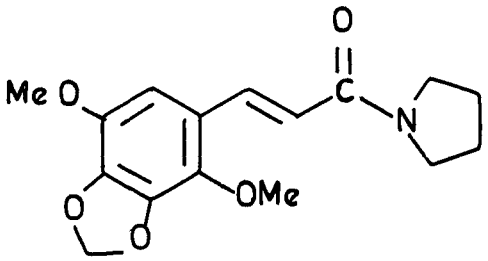
ANOMALIES OF THE METHOD OF BENZENE INDUCED METHOXY PROTON SHIFTS:

The method of methoxy proton shifts, although very useful in structure determination, may lead to erroneous assignments if not used with caution. The following criteria[120] have been laid down for an appropriate use of the method:

1. The method should not be used directly for compounds containing phenolic groups. It was found misleading as in the assignment of an incorrect structure to mangostin.[121,122] The methoxy proton shifts in lonchocarpan[120] were also unexpected.
2. Even acetylation of the phenolic function, as in the case of lonchocarpan acetate,[120] does not completely overcome the difficulty.
3. The fully methylated compounds, are safest to use but even then the results may be misleading if solvation of a separate site close to the methoxy groups being examined occurs. This is shown by large solvent induced shifts of protons other than the methoxy protons. Thus it was not possible to distinguish between two alternative structures (XXXIXa and XXXIXb) for peepuloidin.[123]

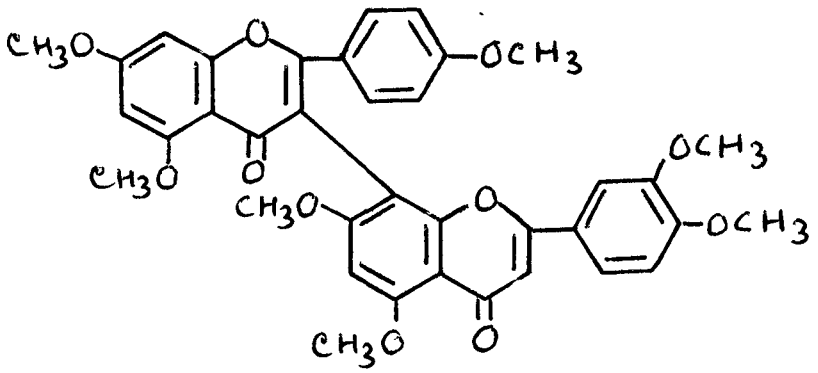


(XXXIX a)



(XXXIX b)

4. In the biflavonyl series[40] the 3"'-methoxy group of WGH-II methyl ether, appears at an exceptionally high position (τ6.56) in $CDCl_3$. This is suggestive of its being entirely internally solvated. A model of this flavone shows that there are in fact certain positions in which that particular methoxy group can be solvated by a benzene ring on the other flavonoid unit thus rendering it unique in being resistant to external solvation. On change of solvent from $CDCl_3$ to C_6H_6 all the methoxy groups were expected to move upfield by more than 30 c/s as each methoxy group had an ortho proton. The methoxy group in question (at τ 6.56), however, moved very little (Fig.VI).



(XL) WGH-II methyl ether

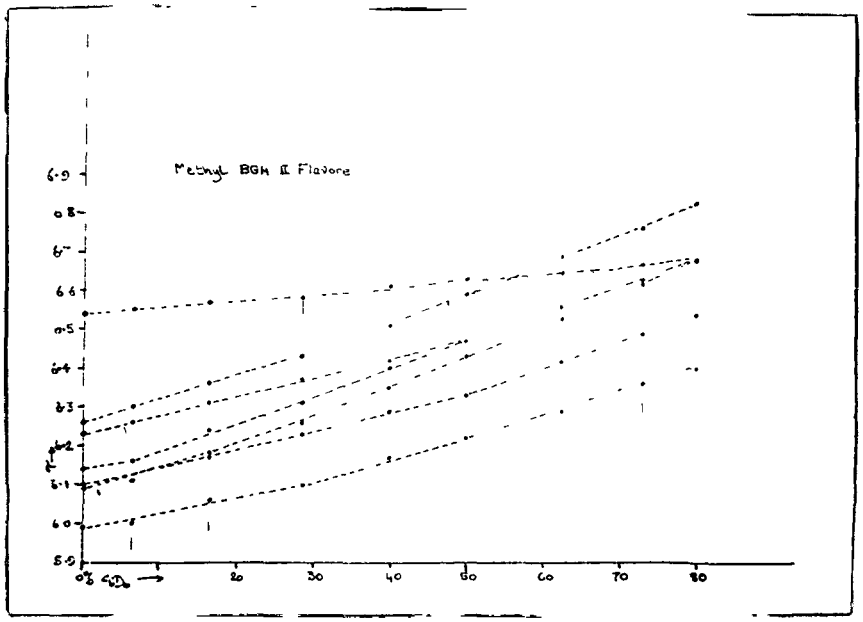

Methyl BGH II Flavone
6.9
6.8
6.7
6.6
6.5
6.4
6.3
6.2
6.1
6.0
5.9
20
30
40
50
60
70
80

Fig. VI

This does mean, of course, that when we are dealing with an aromatic methoxy group in an unusual position, the method of methoxy proton shifts is to be used with the greatest caution.

PROTON ASSIGNMENT

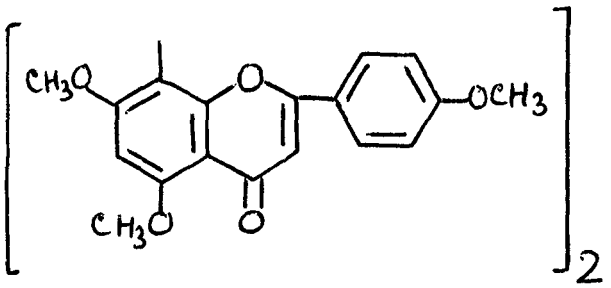
CUPRESSUFLAVONE SERIES:

Due to symmetry of the compound, cupressuflavone and many of its derivatives give comparatively simple spectra.

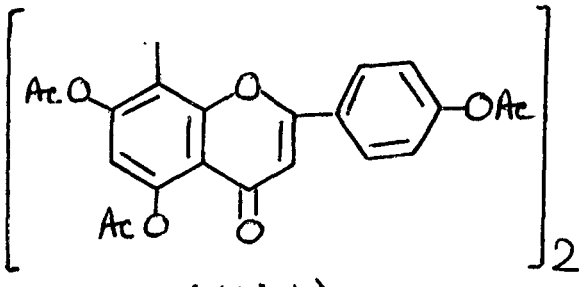
In the parent cupressuflavone and its partial methyl ethers, H-3 is superimposed on H-6 (τ3.43) but in complete methyl ether (XXXII), full acetate (XLI) and the acetates of partial methyl ethers, the pairs may clearly be discerned. The value below τ6.00 (τ5.88) of 5,5"-OMe in cupressuflavone hexamethyl ether (XXXII) may be characteristic of such groups of an 8-8" linked biflavonyl or any 8-linked monoflavonoid unit of a biflavonyl (e.g. amentoflavone, agathisflavone and hinokiflavone). All the methoxy groups above τ6.20 with the exception of 5"-monoacetylpenta-O-methylcupressuflavone are associated with a 4' (4"') grouping.[37] The acetoxy groups at 7(7") always appear above τ7.90 while those at 5(5") and 4'(4"') show up above τ7.45 and τ7.70 respectively. This may be of some value in the orientation of partial methyl ethers.

The limitation of proton assignments in biflavonyls with reference to the NMR data of the corresponding monomers is evidenced by the structure elucidation of dimethyl cupressuflavone as 4',4"'-di-O-methylcupressuflavone[34] which was later revised to 7,7"-di-O-

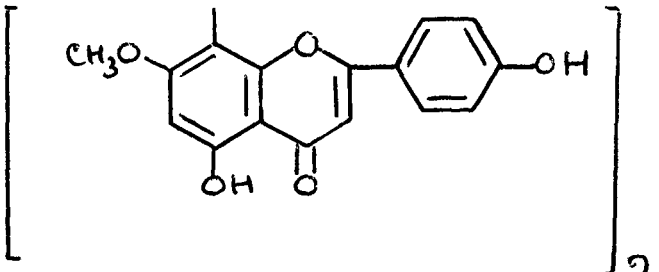
methylcupressuflavone (Ic)[32,33,109,110]



(XXXII)



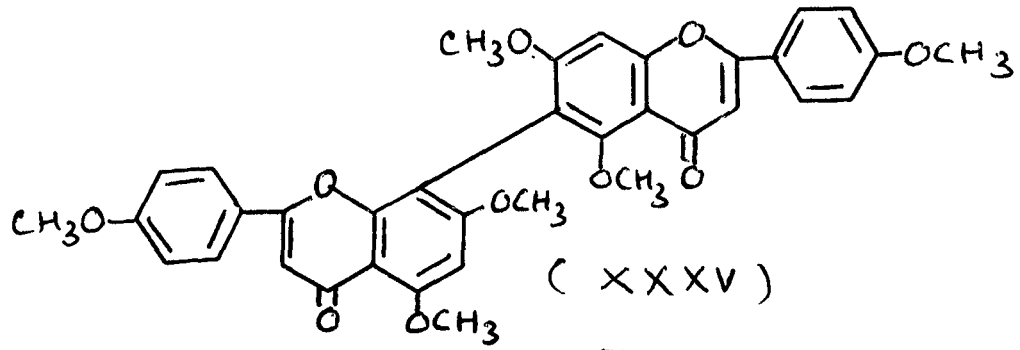
(XLI)



(Ic)

AGATHISFLAVONE SERIES:

NMR spectrum of agathisflavone hexamethyl ether (XXXV) shows conclusively that the molecule is not symmetrical. Consideration



(XXXV)

of various possibilities of linkage[38] leaves only rings A and D implicated in the interflavonyl linkage and as the molecule is not symmetrical the linkage must be C_6-C_8". All that remains now is

the choosing between the 6" and 8-hydrogen, the proton assignment of rings B and A and the allocation of methoxy groups.

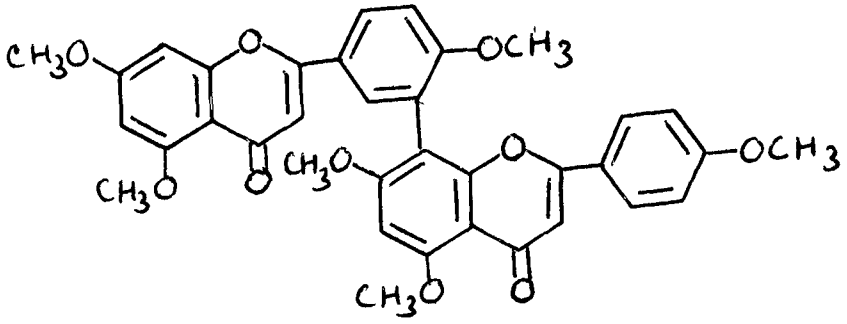
The signal at τ 3.36 (XXXV) is assigned to H-6" by analogy with the chemical shifts of such protons on an 8-linked flavone ring (C-6, τ 3.41) cupressuflavone hexamethyl ether (XXXII)[34].

The signal at τ 3.09 (XXXV) is attributed to H-8 of ring A. This is in line with the report that H-8 of 5,7-dimethoxyflavone[102] is τ 0.20 downfield than H-6. This trend is also maintained in acetates.

The protons assigned to H-2"',6"' (τ2.63) and H-3"',5"' (τ3.22) of ring E (XXXV) fit well by analogy with the similarly constituted E rings of cupressuflavone hexamethyl ether[34] (XXXII), τ 2.62 and τ3.20 and amentoflavone hexamethyl ether[57](XXXIII), τ 2.68 and τ3.28. The protons at τ 2.12 and τ 2.99 are assigned to 2',6' and 3',5' positions respectively of the ring D of the flavonoid unit linked in the hitherto unknown fashion at the 6-position. The above proton assignments to rings B & E are tentative as synthetic evidence to back them is still lacking.

The lowfield value τ 5.95 is characteristic of 5-methoxy group of an 8-linked flavone unit of biflavonyl. Another possibly diagnostic feature is the 5-methoxyl of the 6-linked unit which shows at τ 6.41 (much higher than such methoxyls of any known series of naturally occurring biflavonyls) presumably as it is in the deshielding cone of ring D. It is not possible to distinguish between H-3 and H-3".

AMENTOFLAVONE SERIES :

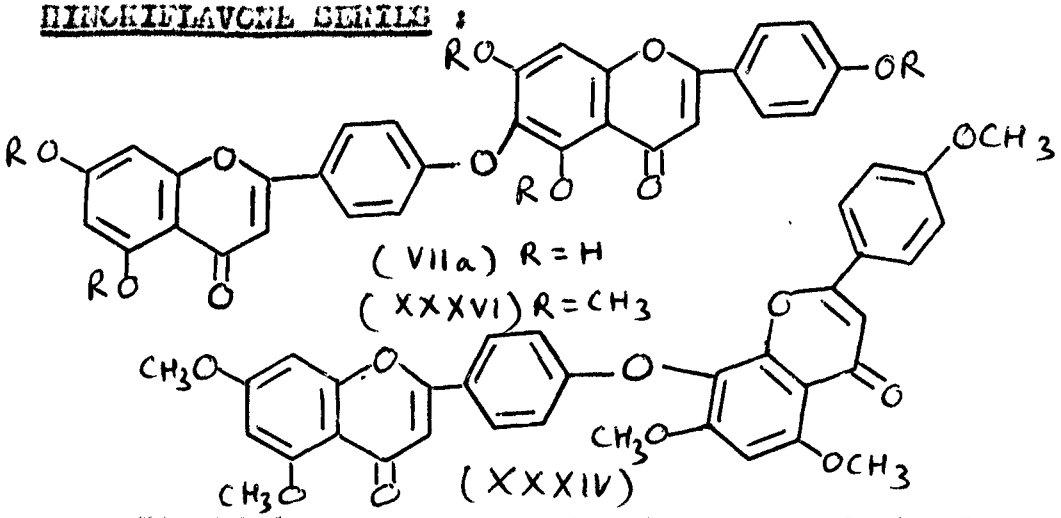


(XXXIII)

In this series, rings B and E may readily be distinguished by the multiplicity of the proton systems on each (ABX and A_2B_2) which can generally be disentangled by the use of double irradiation techniques. For a similar reason rings A and D are clearly marked out, only the protons at C-3 and C-3" being indistinguishable. The H-6" (ring D) cannot be assigned by analogy with the H-6 of the corresponding monomer. It is worth mentioning that the values associated with ring B are very low while ring E remains normal.

A consistent pattern from an overall examination of NMR spectra emerges which may be used for structural assignment in this series.[40-60]

HINOKIFLAVONE SERIES :

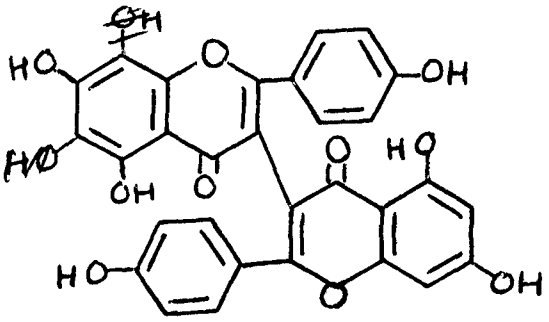


Hinokiflavone was previously assigned the $C_{4'}$-O-$C_{8''}$ linkage (XXXIV) on the basis of spectral and degradative[104,105] studies. The elegant total synthesis of hinokiflavone pentamethyl ether by Nakazawa[124] proved that the compound had the $C_{4'}$-O-$C_{6''}$ linkage (XXXVI) which was later supported by solvent induced shift studies of methoxy resonances.[62] There is large difference between the chemical shift values of H-6" (τ3.49) and H-8" (τ2.95) for the two series.

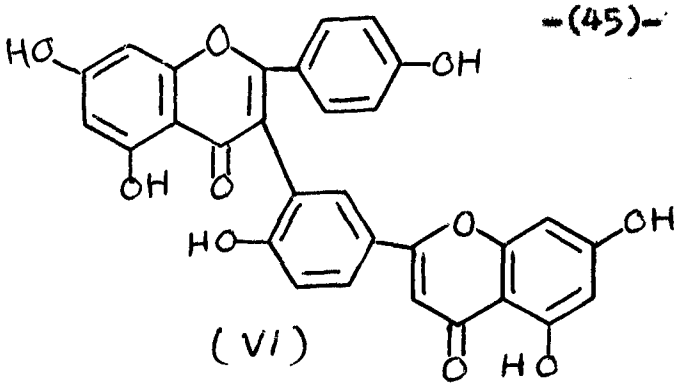
On looking at the proton chemical shifts of the methoxyls, the acetoxyls and the ring protons, certain important correlations emerge which have been found useful in structure elucidation in the series.

3,3" and 3,3"' LINKED BIFLAVONYLS[61]

The proton shifts of methyl ethers and acetates of (V) and (VI) have been helpful to establish their structures as 4',4"',5,5",7,7"-hexahydroxy-3,3"-biflavonyl and 4',4"',5,5",7,7"-hexahydroxy-3,3"'-biflavonyl respectively.[61]



(V)



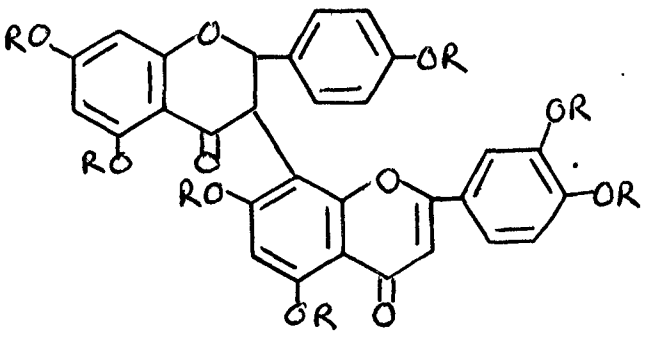
(VI)

BGH SERIES:[39,69-74]

The NMR spectra of BGH-III is discussed with reference to that of BGH-II.[69] The BGH-II methyl ether (XXXVIII) showed methoxy signals between τ6.08 to τ6.36 which integrated for seven methoxyls. The doublets at τ4.16 and τ5.08 (J_{trans}=12 c/s) were shown to be coupled by double resonance. These were assigned to H-2 and H-3 trans aliphatic protons of ring C of the flavanone unit.

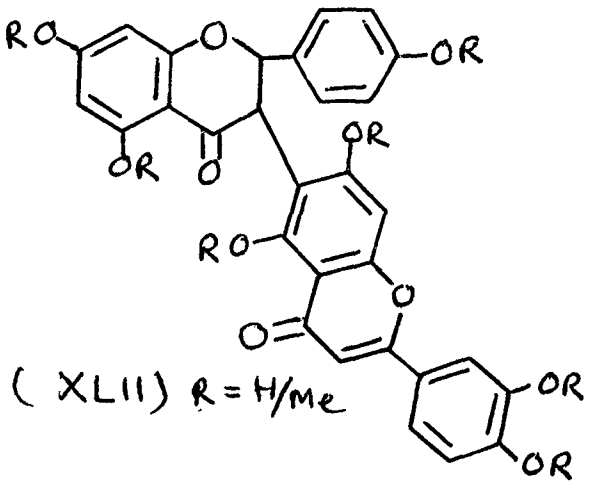
The aromatic protons were assigned as usual.[71]

The data is well consistent with the structures (VIIIa) and (XLII) in which C-3 of the flavanone unit is linked with the C-8 or C-6 of the flavone unit. The possibility of an isoflavanone-



(VIIIa) R=H

(XXXVIII) R = Me



(XLII) R = H/Me

flavone structure involving C2-C8"/C_2-C_6" linkage could not be ruled out at this stage as in the NMR spectrum of methyl ether the

aliphatic protons at C-2 and C-3 both having an aromatic substituent were expected to show the same proton shifts as in the flavanone-flavone structure (VIIIa). The fragmentation pattern of the methyl ether and the ozonolysis of the corresponding chalkone-flavone acetate, however, supported the C_3-$C_{8''}$/C_3-$C_{6''}$ type of linkage. The problem of implication of C-8"/C-6" of the flavone unit in the interflavonyl linkage was solved by studies on solvent induced shifts of methoxy resonances.[40,74]

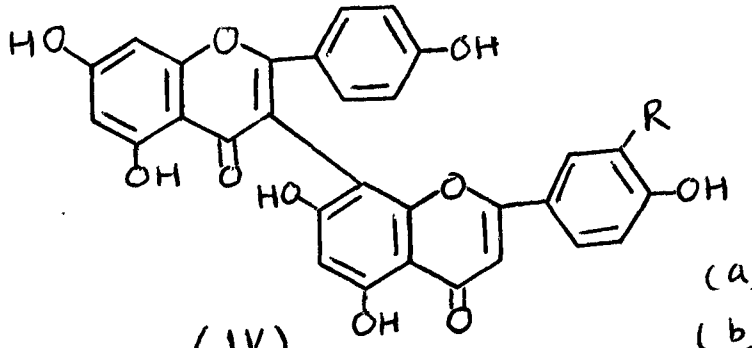
The most interesting observation was the complete lack of correlation between the NMR spectrum of BGH-II acetate (pyridine-acetic anhydride) and its methyl ether.[40]

WGH SERIES (3,8" LINKED BIFLAVONYLS)[40]

The spectra of methyl ethers of WGH-III (IVa) and WGH-II (IVb) were comparable with those of BGH-III and BGH-II methyl ethers respectively except that the aliphatic protons of ring C in the latter had disappeared in the former.

The 2',6' signals both in WGH-III and WGH-II appeared appreciably at lower field than those in BGH-III and BGH-II respectively. This, perhaps may be due to the deshielding effect of the double bond in the pyrone ring upon the B ring protons. The 2"',6"' protons of ring E, on the other hand, moved upfield. The protons at C-6(6") showed a downfield shift. A striking feature of the spectrum of WGH-II methyl ether was the appearance of an aromatic methoxy group at an exceptionally high position (τ 6.56) suggestive of its being already entirely solvated. The interflavonyl linkage

as shown in (IVa) and (IVb) is supported by the solvent induced shift studies.



(IV)

(a) WGH-III , R = H

(b) WGH-II , R = OH

GB SERIES[75a,c,76,77]

The assignment of aromatic protons presents no problem. The doublets at τ 4.38 and τ 5.4 (J_{trans} = 10 c/s) were shown to be coupled by double resonance. These were assigned to H-2" and H-3" trans protons of ring F. Of the other two such protons of ring C, one doublet is shown at τ 5.00 (J_{trans} = 10 c/s) while the other doublet must be at τ 6.2 (from double irradiation method). This is obscured by methoxy groups. Six methoxyls appear in the region τ 6.1-6.3 while one in the heterocyclic ring is shown at τ 6.7. The data is consistent with two flavanone units linked together through C-3 of ring C to either C-6" or C-8" of ring D. The problem of implication of C-6" or C-8" in interflavonyl linkage has not been solved in this series.

The structures of GB-1_a and GB-2 were readily elucidated by comparing the spectral data with that of GB-1. Thus for GB-1_a, which contains one oxygen atom less than GB-1, the NMR spectrum is identical in the aromatic region but has five aliphatic protons.

Two of these are atτ7.3 consistent with the presence of a methylene group at C-3" in ring F in accord with the structure (IXb) for GB-1_a.

BIFLAVONOID GLYCOSIDES :

The NMR data in fukugiside[71], opicatoside[78] and xanthochymusside[78] were in conformity with their respective aglycones. The location of the carbohydrate moiety in the biflavonyl glycosides were accomplished by methylation followed by hydrolysis. The aglycone gave an acetate which showed the presence of one acetoxy group atτ7.82.

NMR CORRELATIONS :

$CDCl_3$ spectra seem reasonably comparable with $(CD_3)_2CO$ spectra though neither with pyridine nor benzene spectra. Certain useful correlations emerge by an examination of NMR data ($CDCl_3$) in biflavonyls. It may be noted that the various series are internally consistent, although there are differences between the series.

1. The methoxy group at C-5 of an O-linked monoflavonoid unit with aromatic ring (A or B) of biflavonyl methyl ethers in all cases examined so far appears belowτ6.00. This may, perhaps, be diagnostic of such constituted units in biflavonyls (Table-V).

TABLE-V

METHOXY PROTON SHIFTS OF COMPLETE METHYL ETHERS

Biflavonyls	5-OMe	5"-OMe
Cupressuflavone	5.85	5.85
Amentoflavone	6.13	5.94
Agathisflavone	6.41	5.95
Hinokiflavone ($C_{4'}$-O-$C_{8''}$)	6.00	5.92
Hinokiflavone ($C_{4'}$-O-$C_{6''}$)	6.06	6.09

Spectra run in $CDCl_3$ on Varian HR 100 spectrometer; TMS as internal standard = τ 10.00.

On change of solvent from $CDCl_3$ to pyridine, it has, however, been observed that such a methoxyl in hinokiflavone methyl ether (4'-O-8") appears above τ 6.00 while that in 4'-O-6" linked biflavonyl below τ 6.00

2. By examining the methoxy and acetoxy shifts, certain useful correlations emerge but they should be used only as supporting evidence. It is by looking at the full series (parent, fully methylated and acetylated products) and comparing multiplicities and positions of the aromatic protons safe assignments can be made.

3. Aromatic protons are completely self-consistent in cupressuflavone, agathisflavone, (assumed values of ring E) amentoflavone and hinokiflavone series. The protons of ring B appear consistently lower than those of ring E.

4. The proton at C-8 in hinokiflavone (C4'-O-C6") and agathisflavone methyl ethers appear at exceptionally low positions τ2.95 and τ3.09 respectively. This may be diagnostic of H-8 (ring A) of a 6-substituted biflavonyl methyl ether both of biphenyl and biphenyl ether types.

5. The proton assignment of biflavonyls with reference to n.m.r. data of the corresponding monomers should be used with caution. This is evidenced in the structure assignment of 4',4"'-di-O-methyl cupressuflavone[34] which was later revised to 7,7"-di-O-methyl cupressuflavone[32,33,102,109,110]

6. The dependency of H-6" of ring D upon its mode of bonding (C-O) with the other half of the biflavone has been observed:

Biflavonyl methyl ether	H-6" (value)	H-8" bonded to :
BGH-III	3.82	Reduced heterocyclic ring (3-8")
BGH-II	3.74	" "
WGH-III	3.55	Heterocyclic ring (3-8")
WGH-II	3.49	" "
Cupressuflavone	3.41, 3.42	Ring A(8-8")
Amentoflavone	3.38	Ring B(3'-8")
Agathisflavone	3.36	Ring A(6-8")

MASS SPECTROSCOPY

The mass spectra of a wide variety of organic natural products have been studied only during the last few years. The inlet system suitable for volatilization of high molecular weight (M^+, 300-1200) organic materials has increased the utility of mass spectroscopy. Generally fragmentation pattern is related to the structure of the intact molecule. Recently a number of papers on the evaluation of structure-fragmentation pattern relationship in mono-and biflavonoid have appeared.[125-128]

FLAVONES:

Unsubstituted flavone (XII)[125] gives the molecular ion as the base peak at m/e 222 (100). The base peak shows subsequent loss of one hydrogen to give an ion, m/e 221 (33) of doubtful structure, while elimination of CO gives ion at m/e 194 (52) (Chart-II, route -A). The fission of heterocyclic ring in flavone, gives two ions, one with a quinonoid, m/e 120 (80) and the other a phenylacetylene m/e 102 (12) structures.

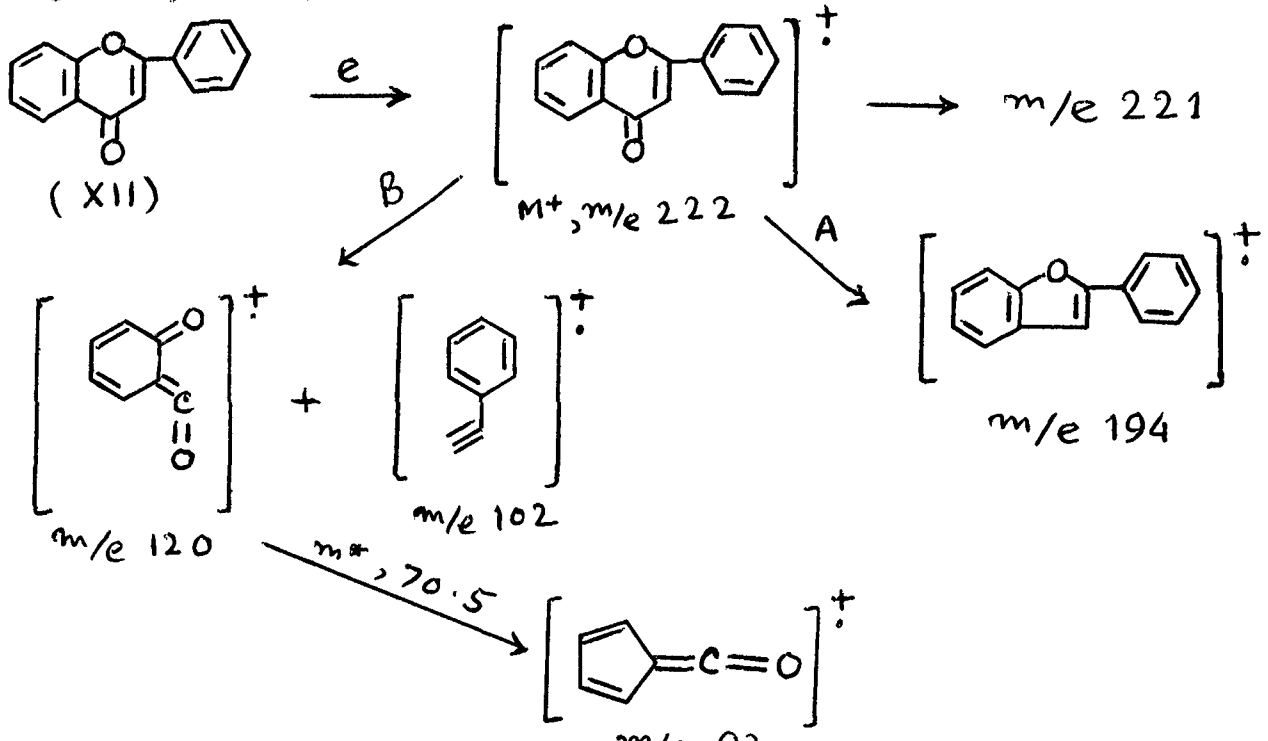


CHART - II

The ion at m/e 120 may lose CO to give the ion at m/e 92 (metastable peak at 70.5) (Chart-II, route-B).

Apigenin[126] (XIX) has the parent molecular ion as base peak, which loses a molecule of carbon monoxide to give a major fragment ion m/e 242. Fragment ions of much less abundance correspond to RDA (Retøro Diels-Alder) fission in the heterocyclic ring.

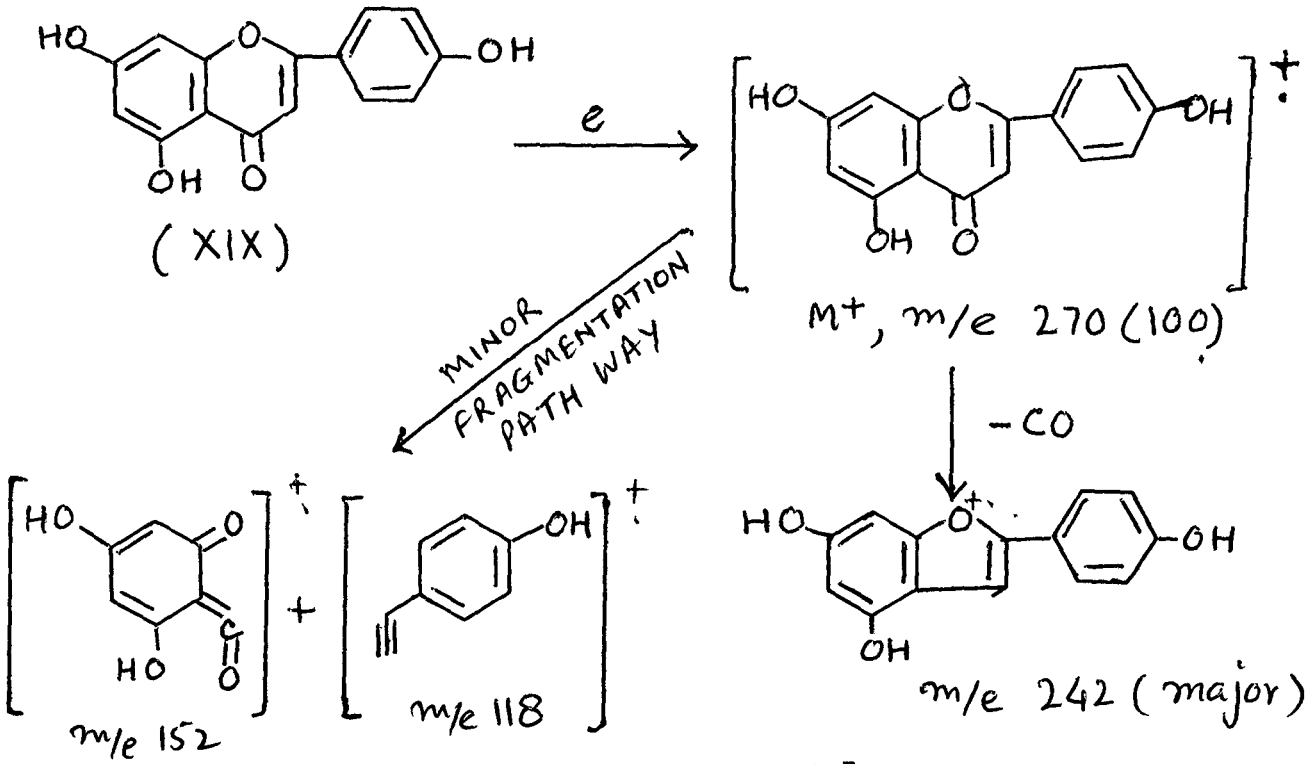


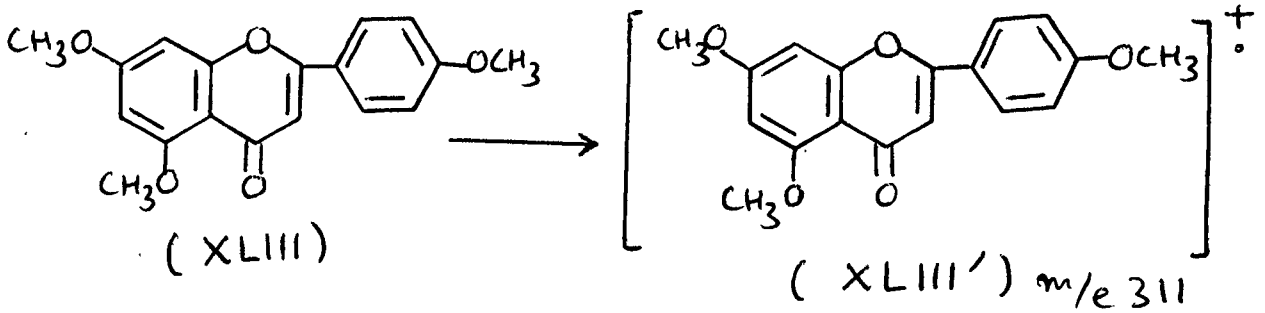
CHART - III

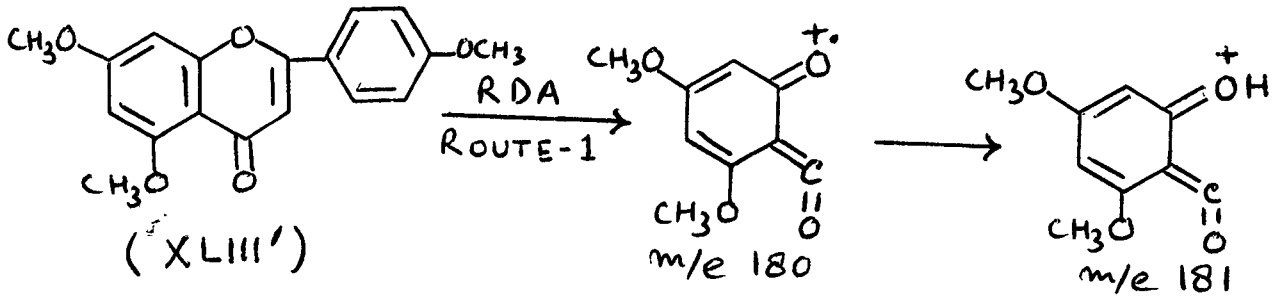
The discrepancy between the results obtained lies in the importance assumed by breakdown via RDA reaction in the natural products as compared with the parent flavone. In the highly oxygenated natural products this fragmentation is minor (15-16% of

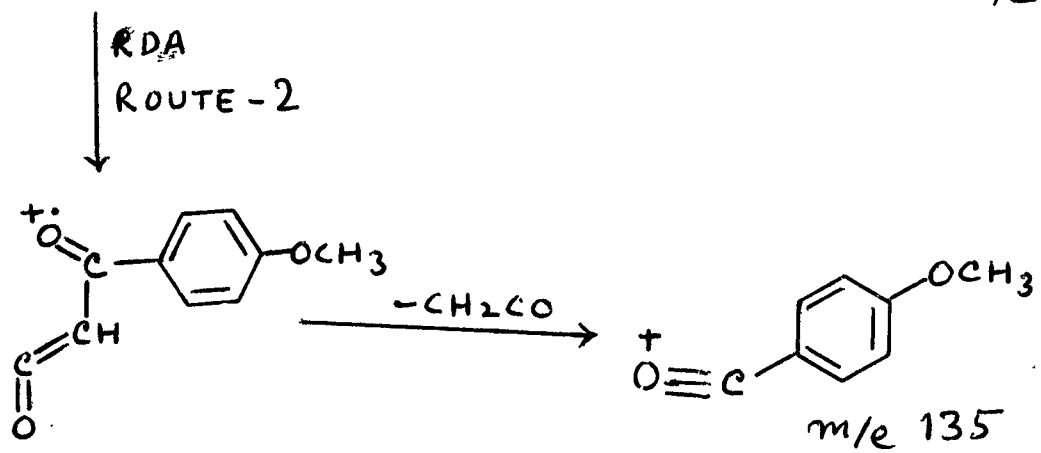
molecular ion) whilst in flavone itself peak due to species with a quinonoid structure is 80% of the intensity of the molecular ion. It appears, therefore, that oxygenation of nucleus profoundly influences the break-down observed. Presumably, if the initially produced ion radical can be stabilized by mesomerism over a number of oxygen atoms, then break-down via RDA is strongly diminished. These minor breakdowns may still prove to be of diagnostic value as they frequently represent the only even numbered peaks in their particular region and hence are readily distinguished.

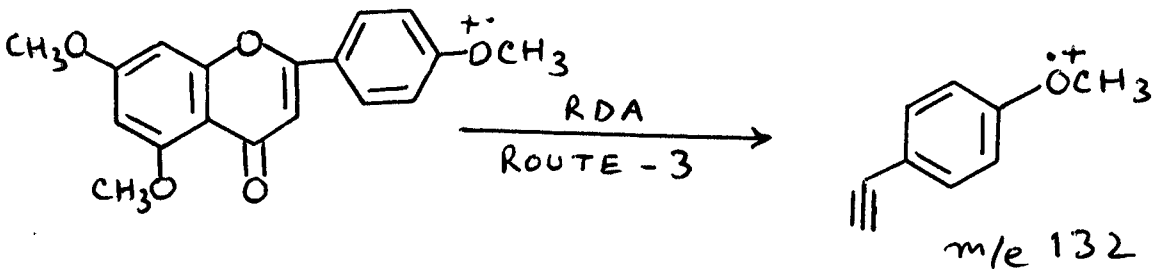
In case of apigenin trimethyl ether (XLIII), the molecular ion appears as the base peak. This is commonly represented either without precisely defining the location of the +ve charge or with the +ve charge localised on the heterocyclic oxygen. Further fragmentation of the molecular ion by the retro-Diels-Alder process yields the ketene (m/e 180), the carbonyl ion (m/e 135) and the acetylene (m/e 132) via routes 1,2 and 3 respectively (CHART - IV)

CHART – IV


(XLIII)
(XLIII′) m/e 311


RDA
ROUTE-1
(XLIII′)
m/e 180
m/e 181


RDA
ROUTE-2
-CH2CO
m/e 135


RDA
ROUTE-3
m/e 132

FLAVANONES:

In case of reduced flavonoids, in which the heterocyclic ring is no longer aromatic, breakdown by paths A and B are of great importance as they lead to clean,cut, characteristic spectra (Chart-V).[128]

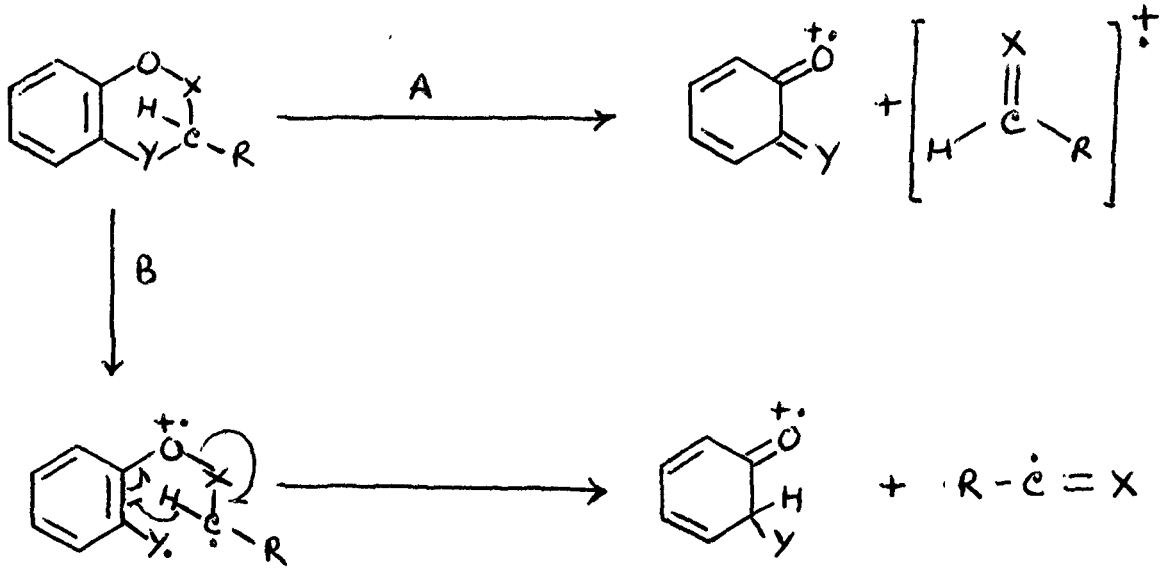
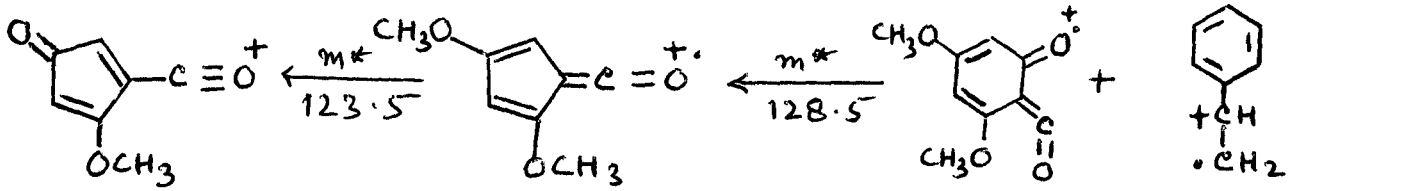


CHART-V

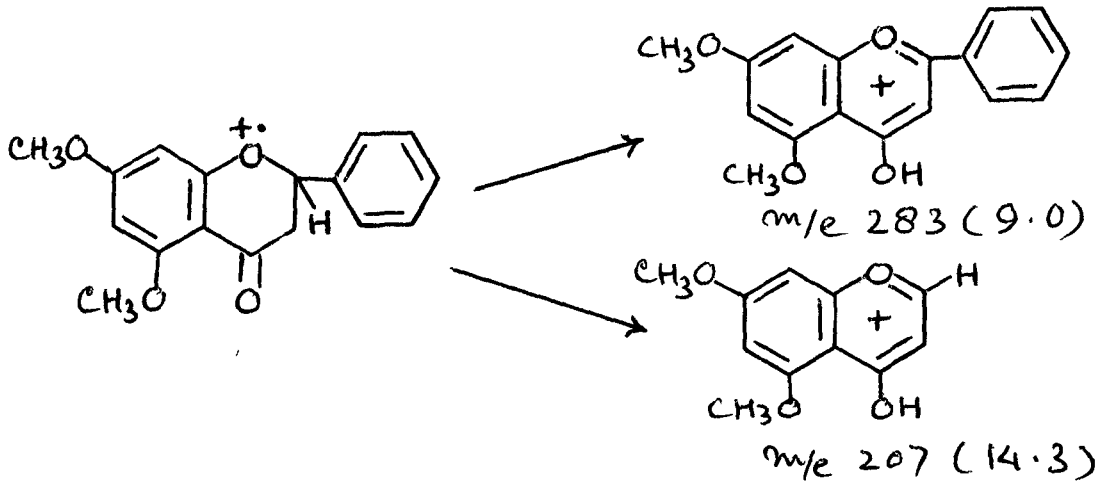
In the mass spectrum of 5,7-dimethoxy flavanone (XLIV),[128] the major pathway involves breakdown by mode A to give the fragments of m/e 180 and m/e 104, the former containing two methoxy groups taking most of the charge. This species loses carbon monoxide to give fragment at m/e 152, the series being terminated by the loss of a methyl radical to give the even electron species at m/e 137.

(XLIV) (XLIV') m/e 284 (51)



m/e 137 (20·0) m/e 152 (21·0) m/e 180 (100) m/e 104 (8·2)

A further method of breakdown, that helps to characterise the flavanones is the loss of either a hydrogen atom or an aryl radical from the molecular ion to give even electron fragments.



m/e 283 (9·0)

m/e 207 (14·3)

A very similar breakdown pattern is found for 4'-methoxy-flavanone (XLV), once more the fragment with methoxyl group taking nearly all the charge. Path B is more noticeable, the breakdown scheme being as shown below(Chart-VI).

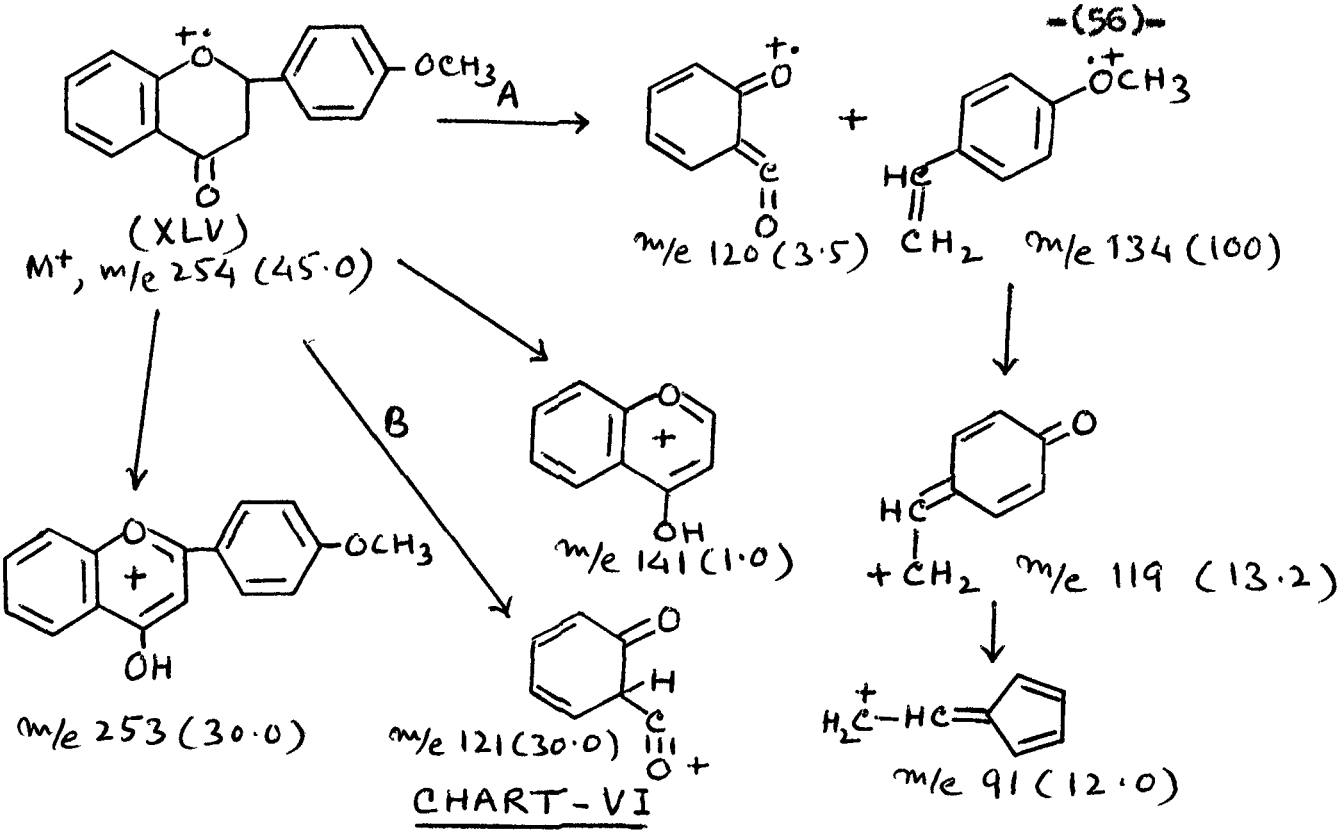
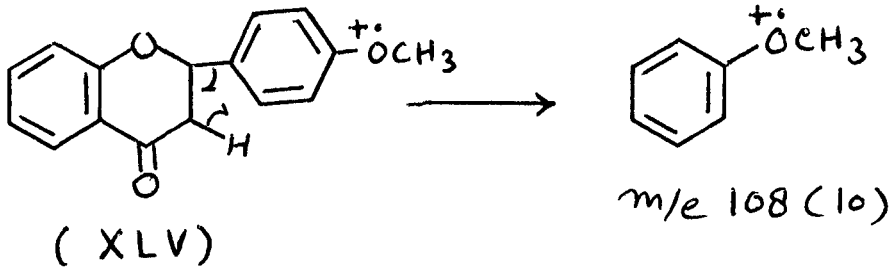


CHART-VI

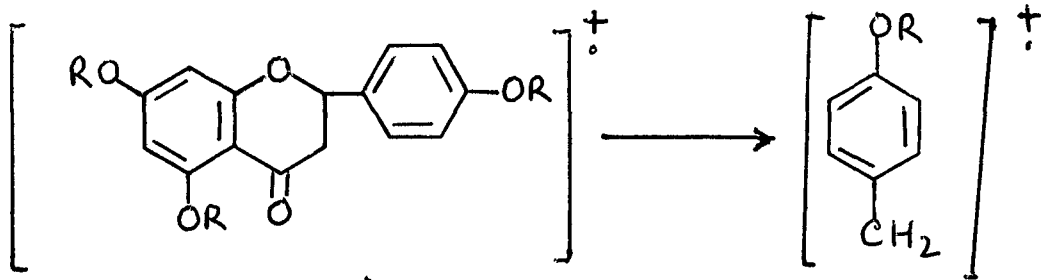
A further peak is at m/e 108 arising from a hydrogen transfer reaction.



(XLV)

The presence of a hydroxyl/methoxyl group at C-4 position of ring B facilitates, by enhanced resonance stabilisation of the resulting fragment ion, the formation of p-hydroxy benzyl/p-methoxy

benzyl ion, which appears as peak of significant intensity in the mass spectrum of naringenin (XLVI)/its trimethyl ether.[128]



(XLVI)

The spectrum of 3,5,7-trihydroxy-4'-methoxy flavanone[128] (XLVII) is of great interest as it was the first reduced flavonoid encountered in which the base peak is neither the molecular ion nor a fragment arising from breakdown via path A. However, this type of break down as well as path B, are found as shown under : (Chart-VII)

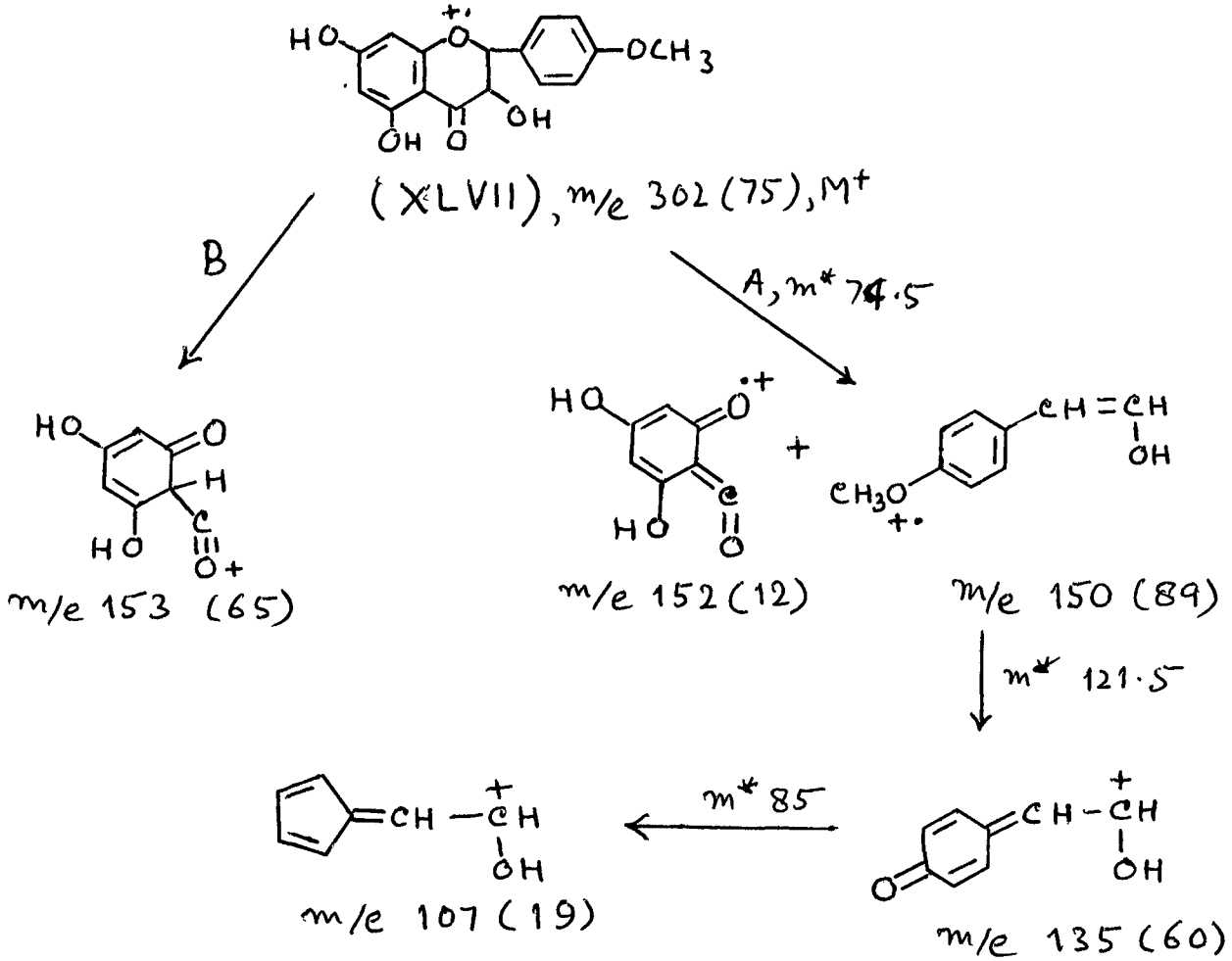
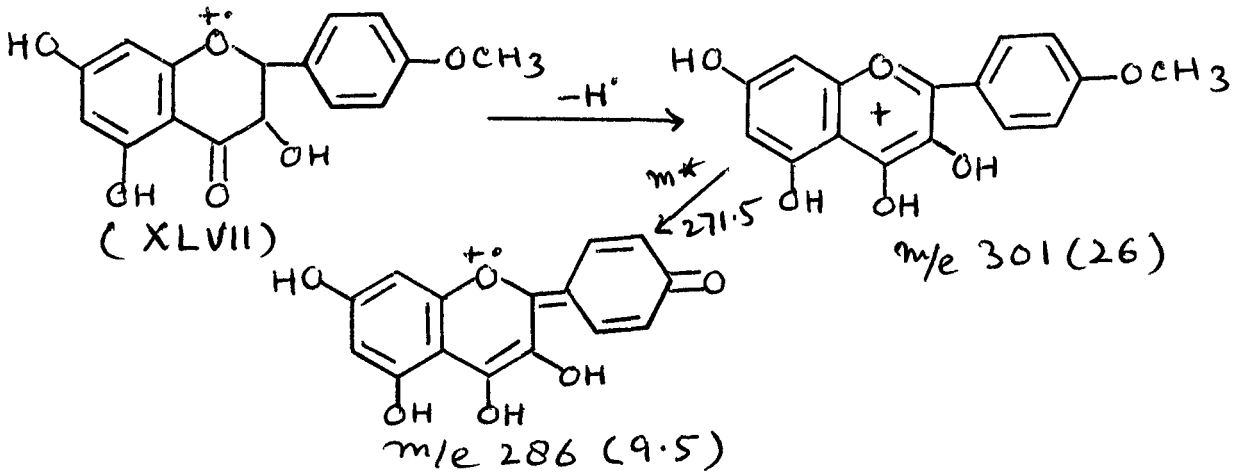
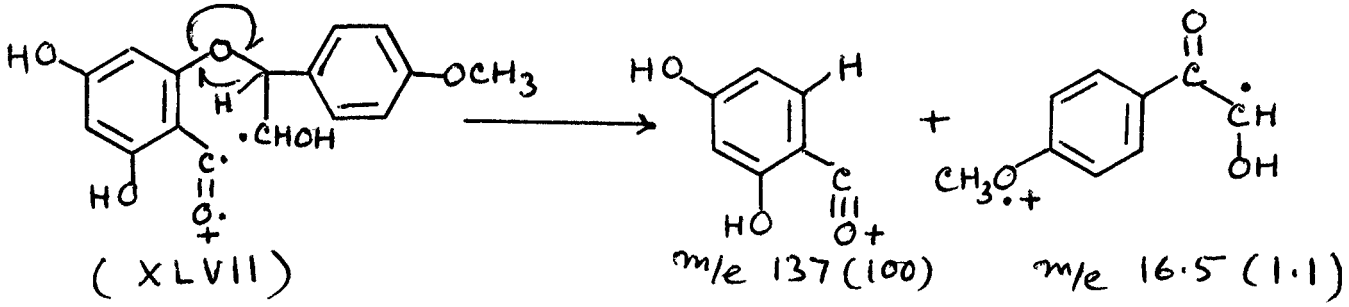


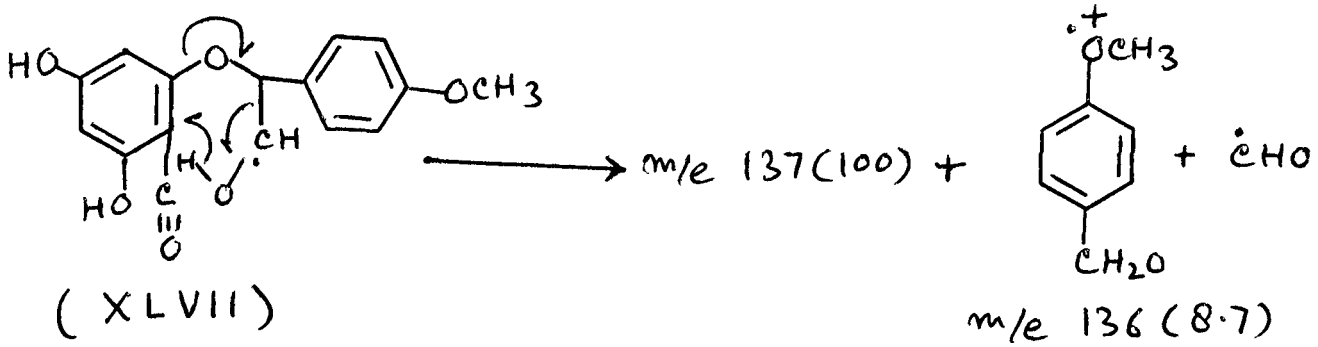
CHART - VII

The loss of a hydrogen atom followed by the loss of a methyl radical is of importance, but the base peak is found at m/e 137. The metastable peak at m/e 62.2 indicates that this fragment is



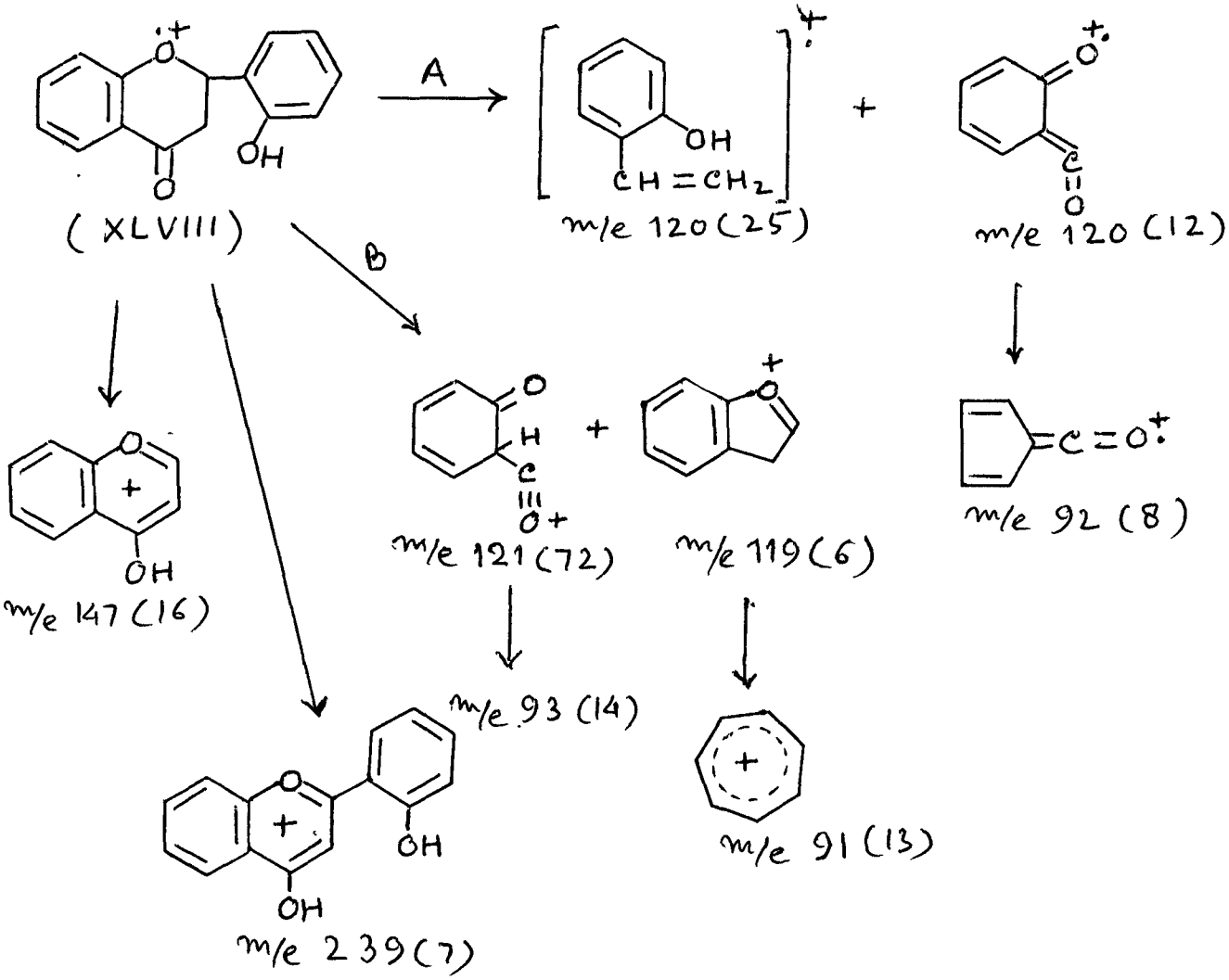
formed directly from the molecular ion. Several processes can give rise to this species.

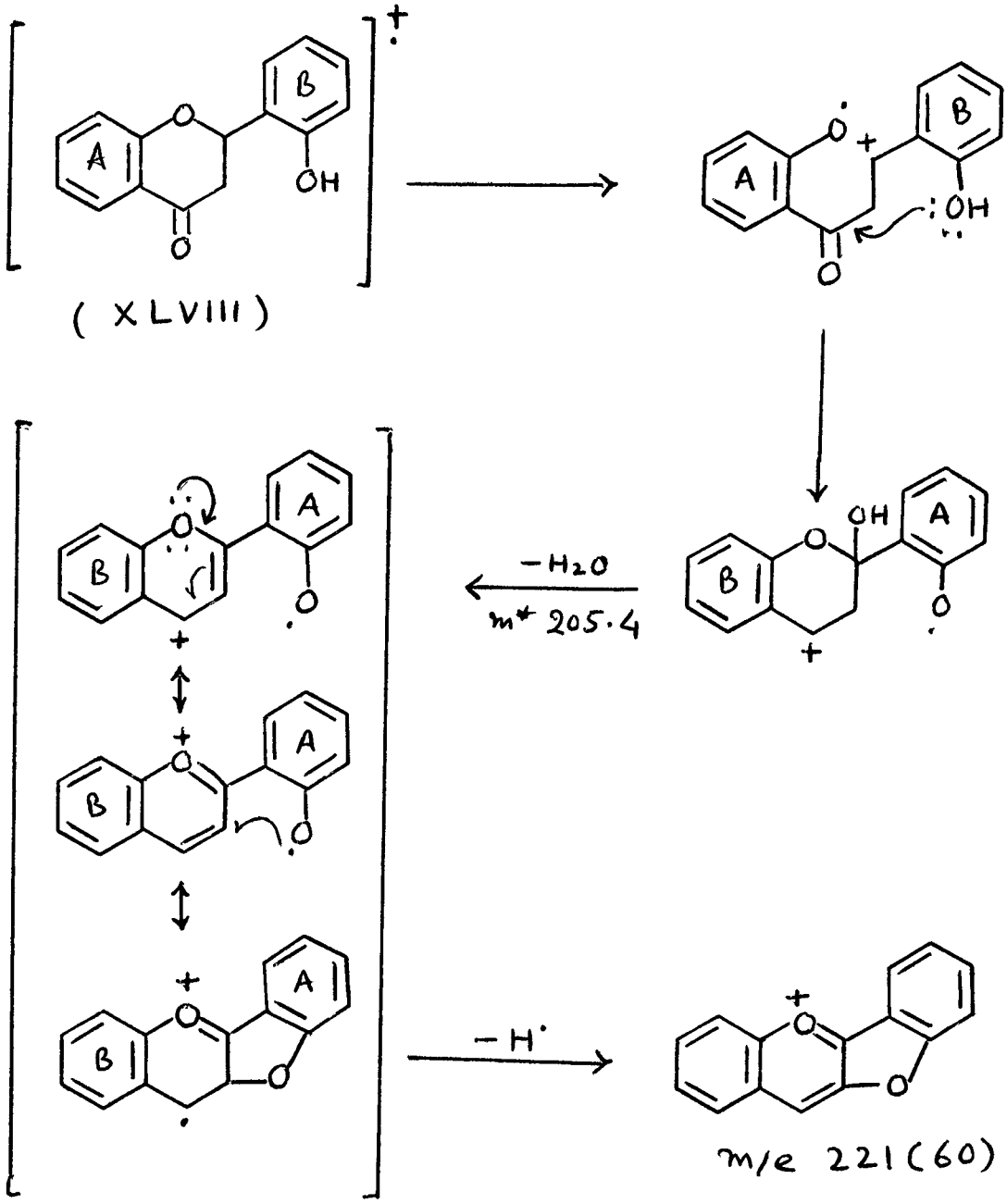




In the case of 2'-hydroxy flavonoids strong intromolecular interactions occur and the breakdown pattern becomes so profoundly modified that it is frequently difficult to classify the substance by reference to standard breakdown patterns.[129]

2'-Hydroxyflavanone[129] (XLVIII) showed breakdown patterns A and B as well as the loss of phenyl or hydrogen radical from C-2 to give even electron species, but the base peak was at M^+-18 and the third largest peak at M^+-19. It has been proposed that these peaks arise by ring opening of the molecular ion followed by ring closure on to the 2'-hydroxy groups as shown in Chart-VIII.





(XLVIII)

CHART - VIII

BIFLAVONOIDS:

The interpretation of the mass spectra of biflavonyls should take into consideration the fragmentation modes of apigenin trimethyl ether[75a,130] and of biphenyls[131a,132,133] or of biphenyl ethers.[131b,c,133]

The fragmentation pattern of biphenyl type biflavonyls viz. cupressuflavone hexamethyl ether and amentoflavone hexamethyl ether are similar, molecular ion being the base peak in each case.[127] Steric factors also seem to play an important role in influencing the breakdown mode and internal condensations. Those factors become so much dominant in agathisflavone hexamethyl ether that the ion at m/e 311 appears as base peak instead of molecular ion, m/e 622 (90).

Cupressuflavone hexamethyl ether (XXXII):

The mode of fragmentation is shown in Chart-IX.

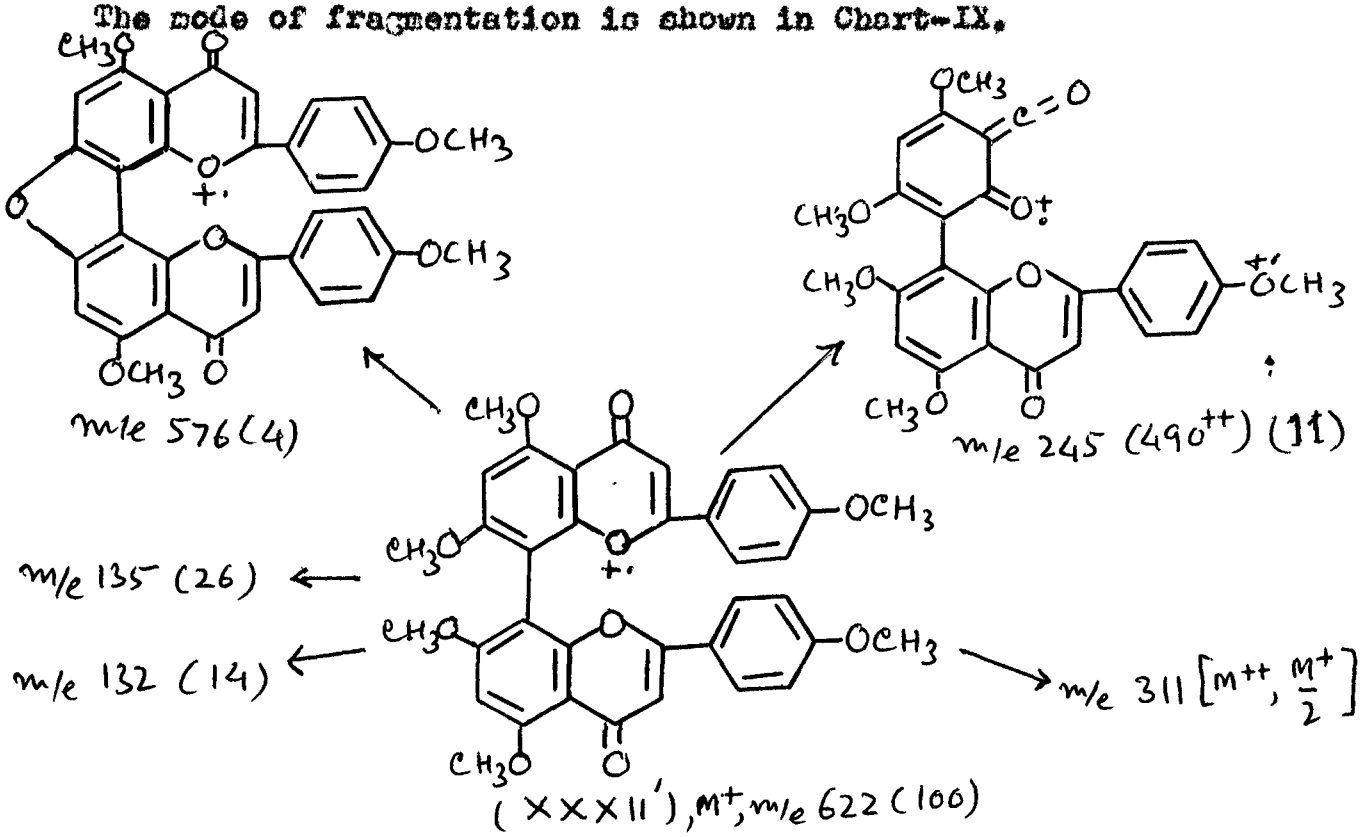


CHART IX

Main peaks:

622(100); 621 (38); 607 (8); 592 (18); 576 (4); 312 (7); 311(14); 245 (11); 135 (26);and 132 (14) (Chart-IX).

Since cupressuflavone hexamethyl ether (XXXII) possesses two apigenin units it is to be expected that the doubly charged molecular ion (M^{++}) will be of considerable intensity with one +ve charge located on one oxygen atom in each of the flavone units. However, the singly charged molecular ion (M^{+}, m/e 622) appears as the base peak.

It is surprising that ketene fragment at m/e 180 which should be expected as a result of the RDA fission of apigenin trimethyl ether unit, is practically absent in the spectrum of cupressuflavone hexamethyl ether.[127]

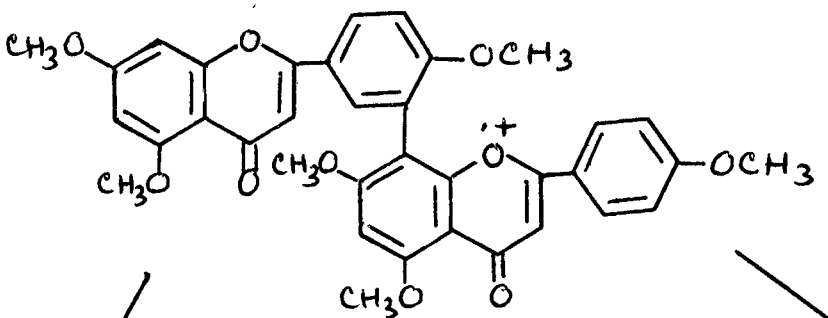
Amentoflavone hexamethyl ether (XXXIII) :

The mode of fragmentation is given in Chart-X.

Main peaks:

622 (100); 621 (31); 607 (33); 592 (8); 576 (10); 312 (2); 311 (5); 245 (5); 135 (16) and 132 (8) (Chart-X).

Amentoflavone hexamethyl ether spectrum shows a peak at m/e 576 (10). In this context it is of interest to note that De Modica et al[134] have reported the formation of such a condensation product (XLIXb) when amentoflavone(IIIa)is heated with zinc dust. An important reason for difference in the intensities of such ions

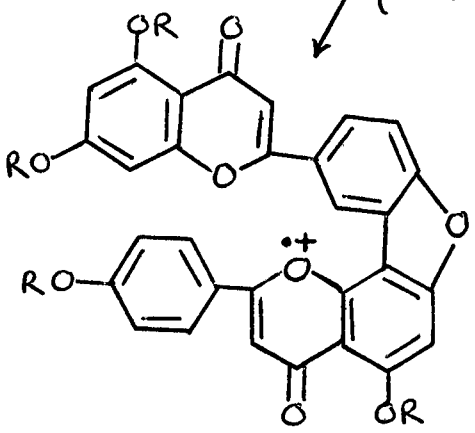


(XXXIIIa) m/e 622 (100)

m/e 132 (3)

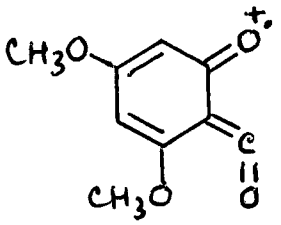
m/e 135 (16)

m/e 311 (5) [M^{++}, $\frac{M^+}{2}$]

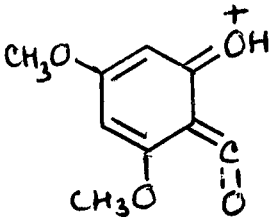


(XLIX) (a) R = CH_3, m/e 576

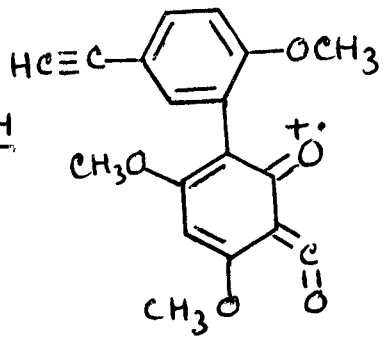
(b) R = H



m/e 180 (3)

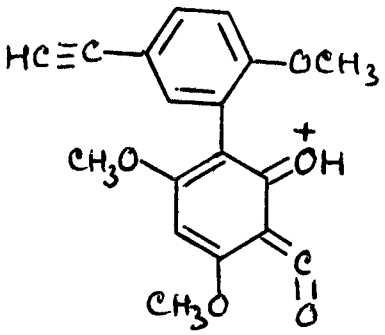


m/e 181 (2)



(L), m/e 310

+H



m/e 311

CHART - X - FRAGMENTATION PATTERN OF AMENTOFLAVONE HEXAMETHYL ETHER

in the spectra of cupressuflavone hexamethyl ether (4%) and amentoflavone hexamethyl ether (10%) is that the former is a symmetric type. This results in differences in the steric disposition of one flavone unit relative to the other, thus hindering or favouring condensation between the phenyl rings.[127]

The main differences in the fragmentation pattern of amentoflavone hexamethyl ether and cupressuflavone hexamethyl ether are in the intensities of the corresponding peaks due to variations in the substitution patterns and steric factors.

The difference in the relative abundance of the ion at m/e 311 (M^{++}, $M^{+}/2$) in the spectra of cupressouflavone hexamethyl ether (XXXII) (14%) and amentoflavone hexamethyl ether (XXXIII) (5%) are of significance and could be explained as follows:-

The variation in the oxygenation pattern of the biphenyl residues in these compounds is responsible for differences in the labile nature of the interapigeninyl bond which consequently leads to differences in the relative intensities of the ion at m/e 311.[127]

Another explanation is also possible for the origin of the peak at m/e 311 in the case of (XXXIII). Double RDA fission of (XXXIII) yields the fragment (L) m/e 310 (3) which accepts a hydrogen atom leading to the ion (La) having m/e 311. Since this ion has the same elemental make up ($C_{18}H_{15}O_5$) as $(M/2)^{+}$ it would not be possible to distinguish between them by accurate mass determination.

Hinokiflavone pentamethyl ether (XXXVI):

The mode of fragmentation is given in Chart-XI.

Main peaks:

608 (39); 607 (12); 593 (36); 580 (4); 579 (11); 578 (11); 576 (6); 431 (7); 327 (23); 313 (100); 312 (22); 311 (22); 304 (2); 297 (29); 296 (75); 281 (22); 181 (11); 180 (3); 135 (19) and 132 (18) (Chart-XI).

The mode of fragmentation of biphenyl type biflavonyls viz. hinokiflavone pentamethyl ether (XXXVI) is considerably different from those of biphenyl ether type. The base ion appears at m/e 313 and the molecular ion (m/e 608) (or its variation) amounts to 39% of this peak. This could be attributed to the fact that the biphenyl ether bridge in (XXXVI) suffers easy rupture; hydrogen transfer then leads to the 313 fragments. The fission of the ether bridge in (XXXVI) can take place in two ways; (i) by route-1 giving the ions at m/e 297 (29) and m/e 311 (22) and (ii) by route-2 giving ions at m/e 281 (22) and m/e 327 (23). However, the observation that the 313 ion is most intense suggests that route 1 is favoured; i.e. the bond between the oxygen bridge and the highly oxygenated phenyl ring breaks preferably.[127]

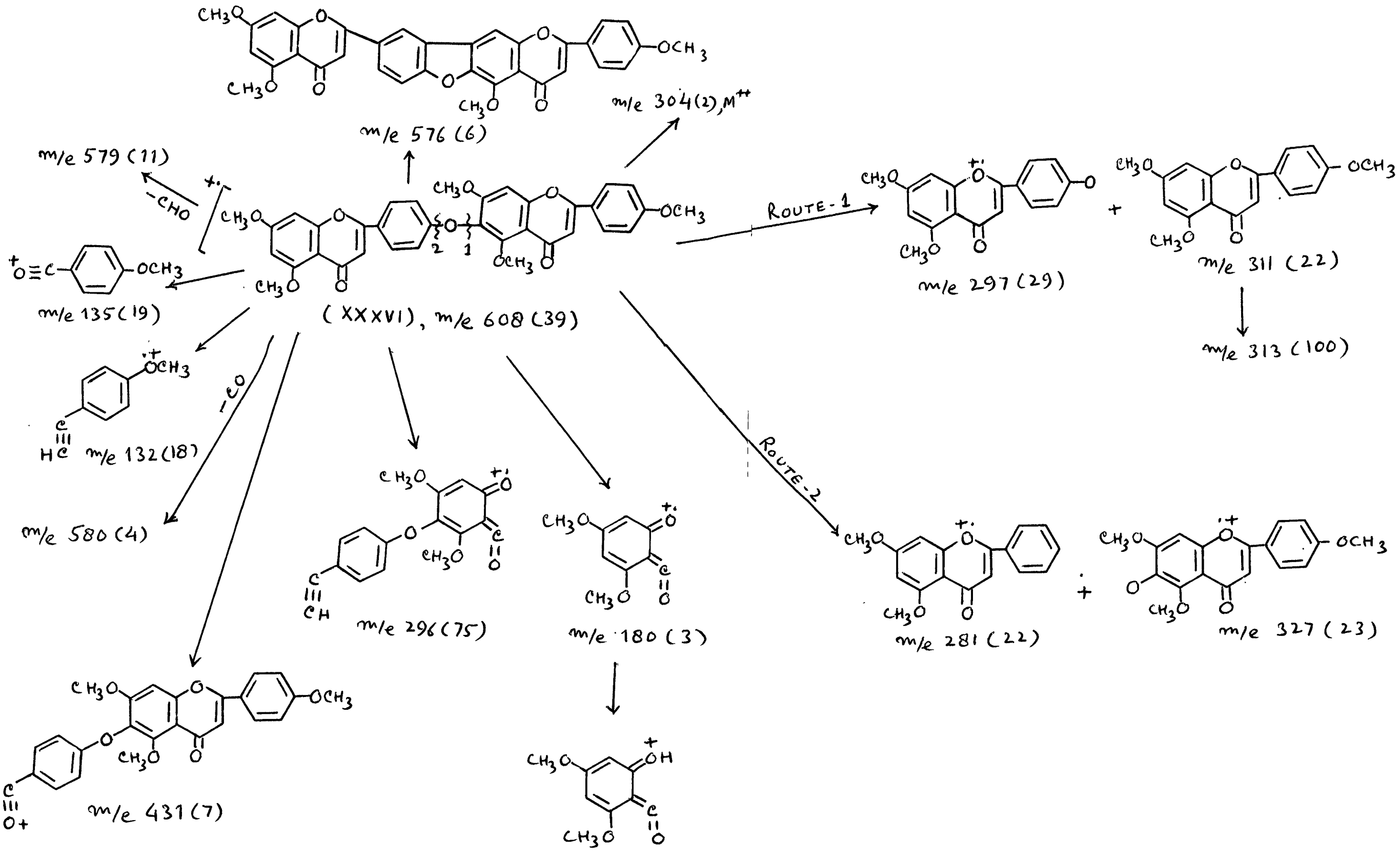
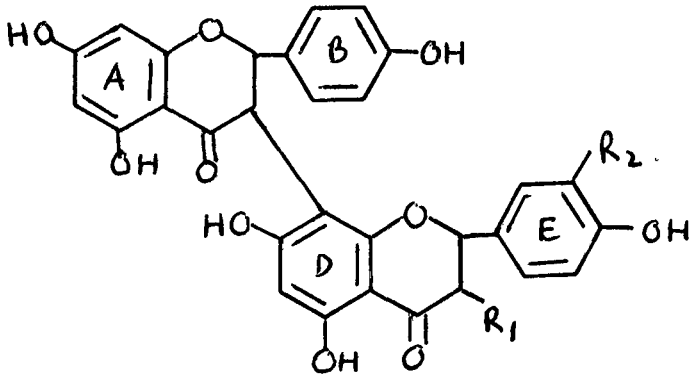

m/e 576 (6)
m/e 304 (2), M++
m/e 579 (11)
-CHO
m/e 135 (19)
(XXXVI), m/e 608 (39)
ROUTE-1
m/e 297 (29)
m/e 311 (22)
m/e 313 (100)
m/e 132 (18)
-CO
m/e 580 (4)
m/e 296 (75)
m/e 180 (3)
ROUTE-2
m/e 281 (22)
m/e 327 (23)
m/e 431 (7)

CHART- XI

GB SERIES (FLAVANONE-FLAVANONE):

The mass spectroscopy has been used by Jackson et al[75a,9] to elucidate the structure of biflavonoids of GB series containing two flavanone units linked through C_3-$C_{8''}$ (IXa-d).



(IX)

(a) GB_1 R_1= OH, R_2= H

(b) GB_{1a} R_1= R_2= H

(c) GB_2 R_1= H, R_2= OH

(d) GB_{2a} R_1= R_2= OH

The mass spectrum of GB_1 heptamethyl ether (LI) (M^+, m/e 656) showed the presence of ions at m/e 121, 154, 181, 312 and 476. The presence of ions at m/e 154 and 181 consistent with the fragments $[C_6H_3(OMe)_2\ OH]^+$ and $[C_6H_2\ (OMe)_2\ OHCO]^+$ supported the presence of phloroglucinol ring system derived from 5,7-dihydroxyflavanone. In addition the presence of another aromatic ring was suggested by an ion at m/e 121 consistent with a $[MeO\ C_6H_4CH_2]^+$ fragment. Mass spectrum also supported the nature of the linkage since the

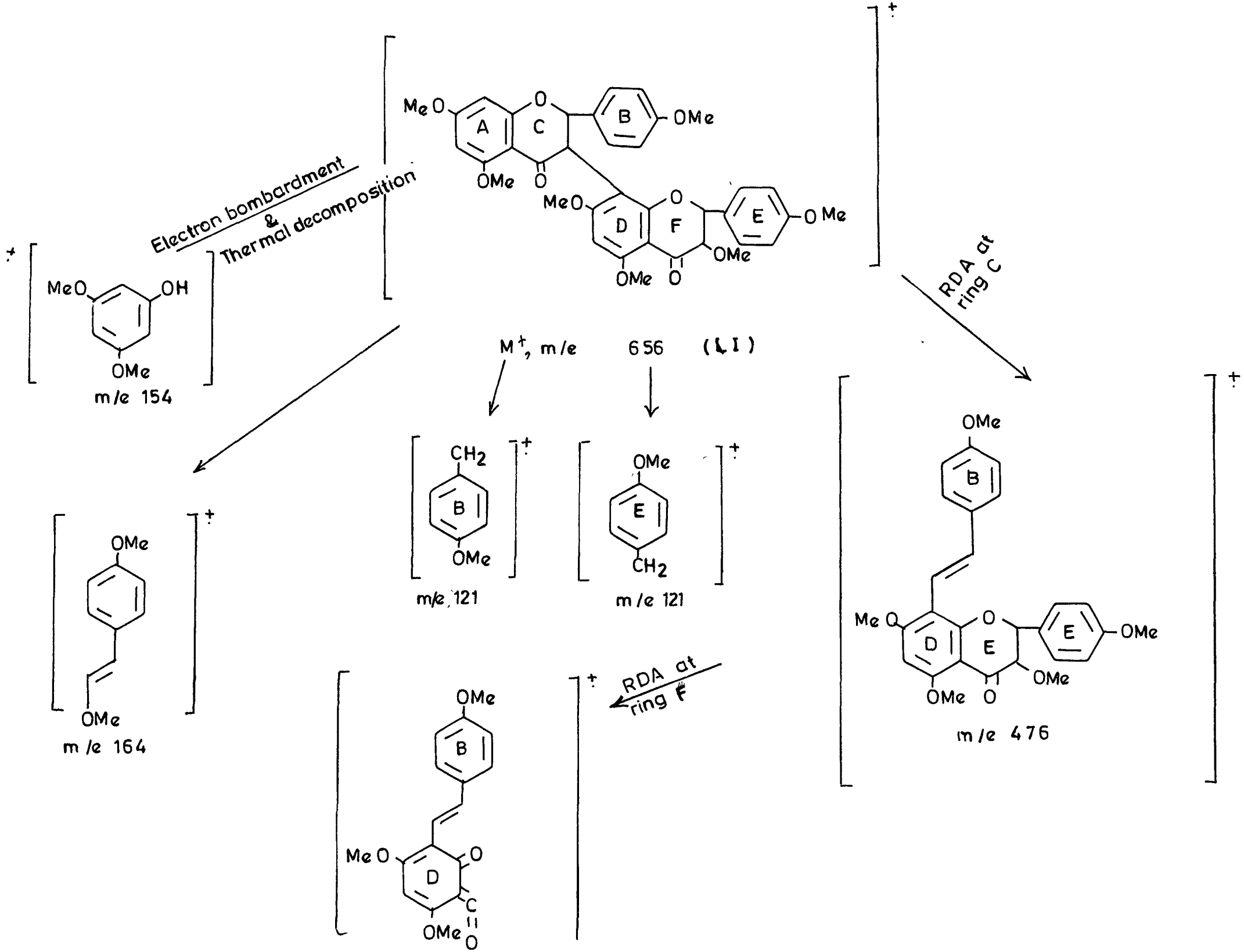
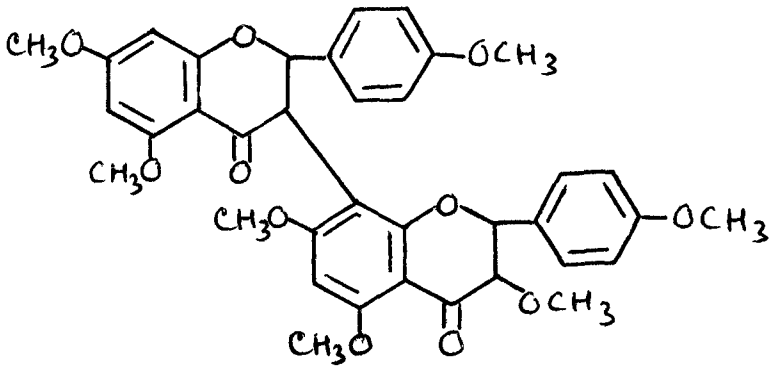

Electron bombardment
&
Thermal decomposition
MeO
OH
OMe
m/e 154
M+, m/e 656 (LI)
RDA at ring C
RDA at ring F
CH2
m/e 121
m/e 164
m/e 476

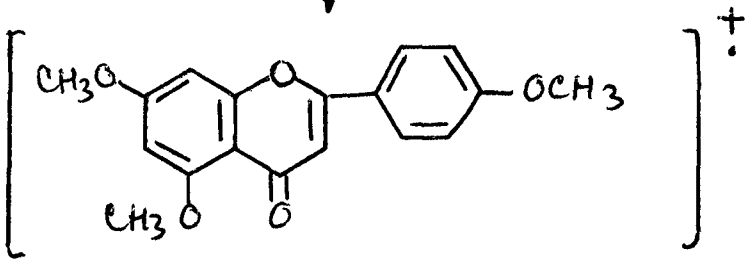
CHART XII

fragmentation of molecular ion at m/e 656 can be rationalised by RDA reaction of flavanones first at ring C to give a fragment ion at m/e 476 followed by a similar fragmentation at ring F to give an ion at m/e 312. This two stage breakdown fragmentation pattern is fully substantiated by the presence of metastable peaks. These results can only be accommodated by a linkage from the oxygen heterocyclic ring C to the phloroglucinol ring D (Chart-XII).

Pelter[76] suggested that the production of fragment ion at m/e 312 can be explained through another mode of fragmentation, while the formation of most of other ions is explainable by RDA reaction characteristic of flavanones (Chart-XIII).



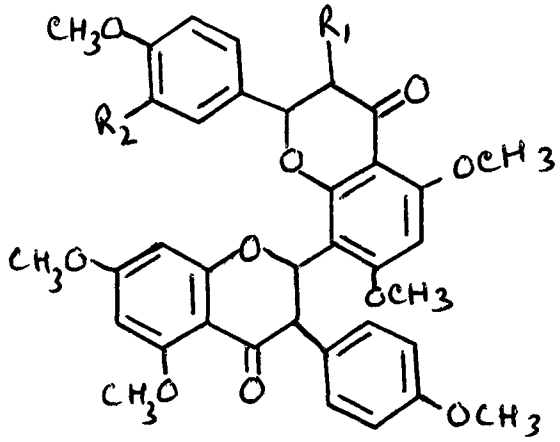
(LI) M^+, m/e 656



m/e 312

CHART - XIII

The production of dimethyl phloroglucinol ion (m/e 154) seems quite odd as simple analogous flavanones do not show this ion. On the basis of previous findings, Pelter further proposed another structure comprising of isoflavanone-flavanone units linked through C_2-C_8" for biflavonoids of GB series.



(LII)

(a) GB_{1a} R_1= R_2= H

(b) GB_1 R_1= OCH_3, R_2= H

(c) GB_{2a} R_1= R_2=OCH_3

(d) GB_2 R_1= H, R_2= OCH_3

The chemical degradation would proceed equally well for compounds of the structures (LIIa-d), this including the oxidation of GB_1 with iodine and sodium acetate to give a biflavonoid type compound. Again NMR spectra would not be able to distinguish the two type of compounds, in each case ring C being substituted at C_2 & C_3 by an aromatic ring. In the mass spectrum of GB_{1a} heptamethyl ether all the ions would be produced by the same process and would have the same structures with the exception of an ion at m/e 312 which would have an isoflavone type structure instead of a flavone type, these being indistinguishable in mass spectrometer. The only spectroscopic evidence that may have any bearing on the

matter is the ion at m/e 121 in the mass spectrum of GB_1 methyl ether. If this is assumed to arise from C_2 of a flavanone, then its presence or absence in GB_2 heptamethyl ether might be indicative of the structure.

The two stage breakdown fragmentation is fully substantiated by the presence of metastable peaks and fragmentation pattern below m/e 312 bears no resemblance to that found in apigenin measured under identical instrumental conditions.[75c] The prominent ions at m/e 180 and 132 noted in the mass spectrum of apigenin trimethyl ether are entirely absent from the mass spectrum of GB_1 heptamethyl ether. This clearly indicates the unacceptability of Pelter's implications.[75c]

The formation of dimethyl phloroglucinol from the methyl ethers is probably of thermal origin. Infact, phloroglucinol is so readily lost from GB biflavanones that if the temperature of the ion chamber in the mass spectrometer much exceeds the minimum (200°C) for evaporation of the sample, there is difficulty in detecting the molecular ion. However, under all conditions the base peak was due to phloroglucinol.[75c]

Further the mass spectrum of GB-2 itself provided the answer to the objection of Pelter[76] as it clearly showed the presence of ions at m/e 107 and 123 consistent with the fragments obtained from aromatic rings B and E respectively.[75c]

Since it has been established that 4'-hydroxyflavanone loses a *p*-hydroxy benzyl fragment at C2, it is perhaps significant that no peak consistent with a 2,4,6-trihydroxy benzyl moiety expected from the alternative structure suggested by Pelter[76] appears in any of the mass spectra of GB series.[75c]

On this basis, Jackson's structure i.e. flavanone-flavanone units linked together is preferable to the other structure i.e. isoflavanone-flavanone. Recent degradative studies on GB biflavonyls[77] further supported structure proposed by Jackson et al.[75a]

Fragmentation pattern of morelloflavone,[69] C_3-C_8" linked flavanone-flavone units is shown in Chart-XIV.

Thus mass spectral studies of the biflavonyls isolated from natural sources reveal that their fragmentation patterns depend not only on the constituent monomeric flavonoid units but also on the nature and the position of interflavonyl linkages. While the cracking patterns of simpler flavonoids are less complex, in application of these concepts to biflavonoids one has to take into consideration the influence of the additional structural and steric factors.

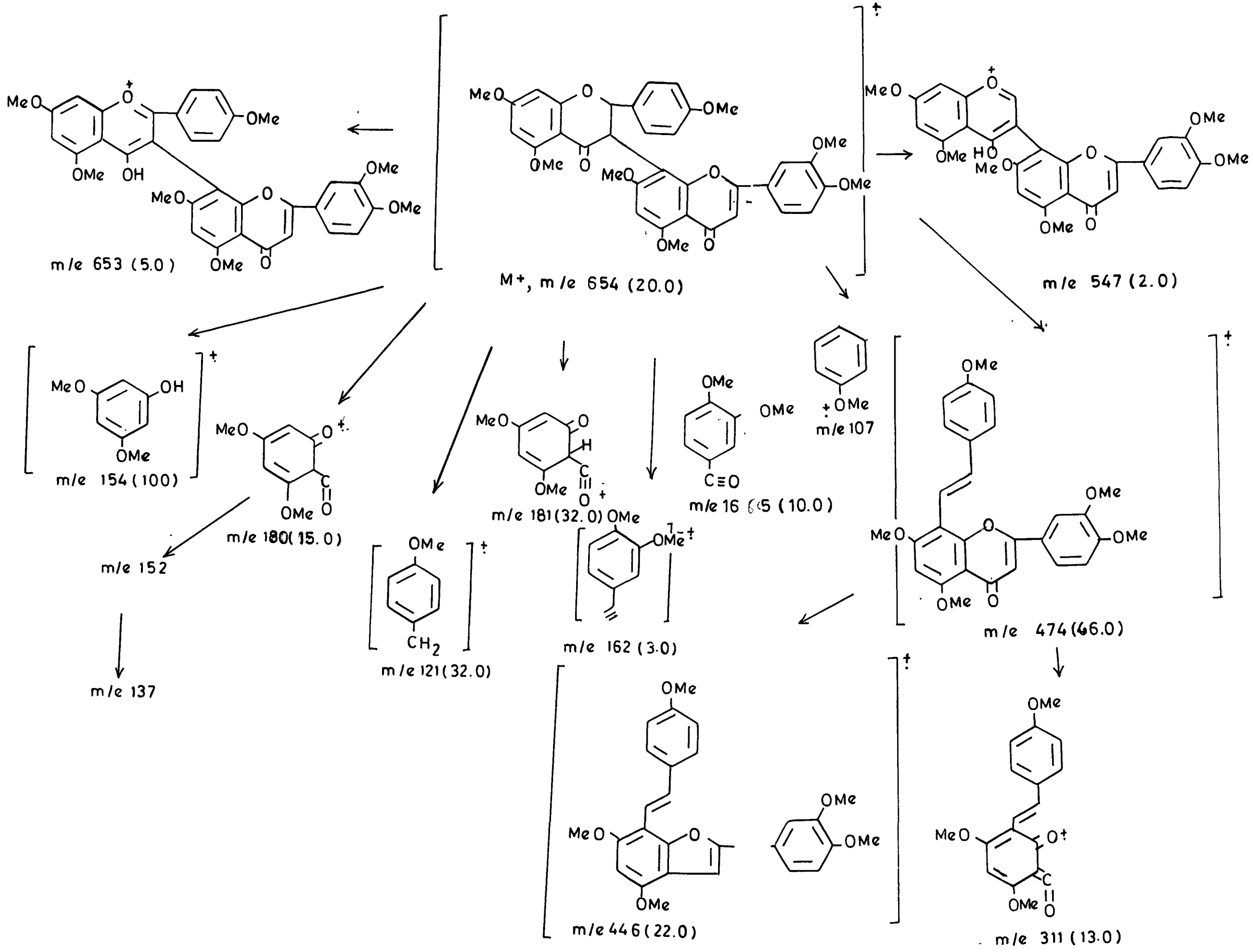

m/e 653 (5.0)
M+, m/e 654 (20.0)
m/e 547 (2.0)
m/e 154 (100)
m/e 180 (15.0)
m/e 181 (32.0)
m/e 165 (10.0)
m/e 107
m/e 152
m/e 137
m/e 121 (32.0)
m/e 162 (3.0)
m/e 474 (46.0)
m/e 446 (22.0)
m/e 311 (13.0)

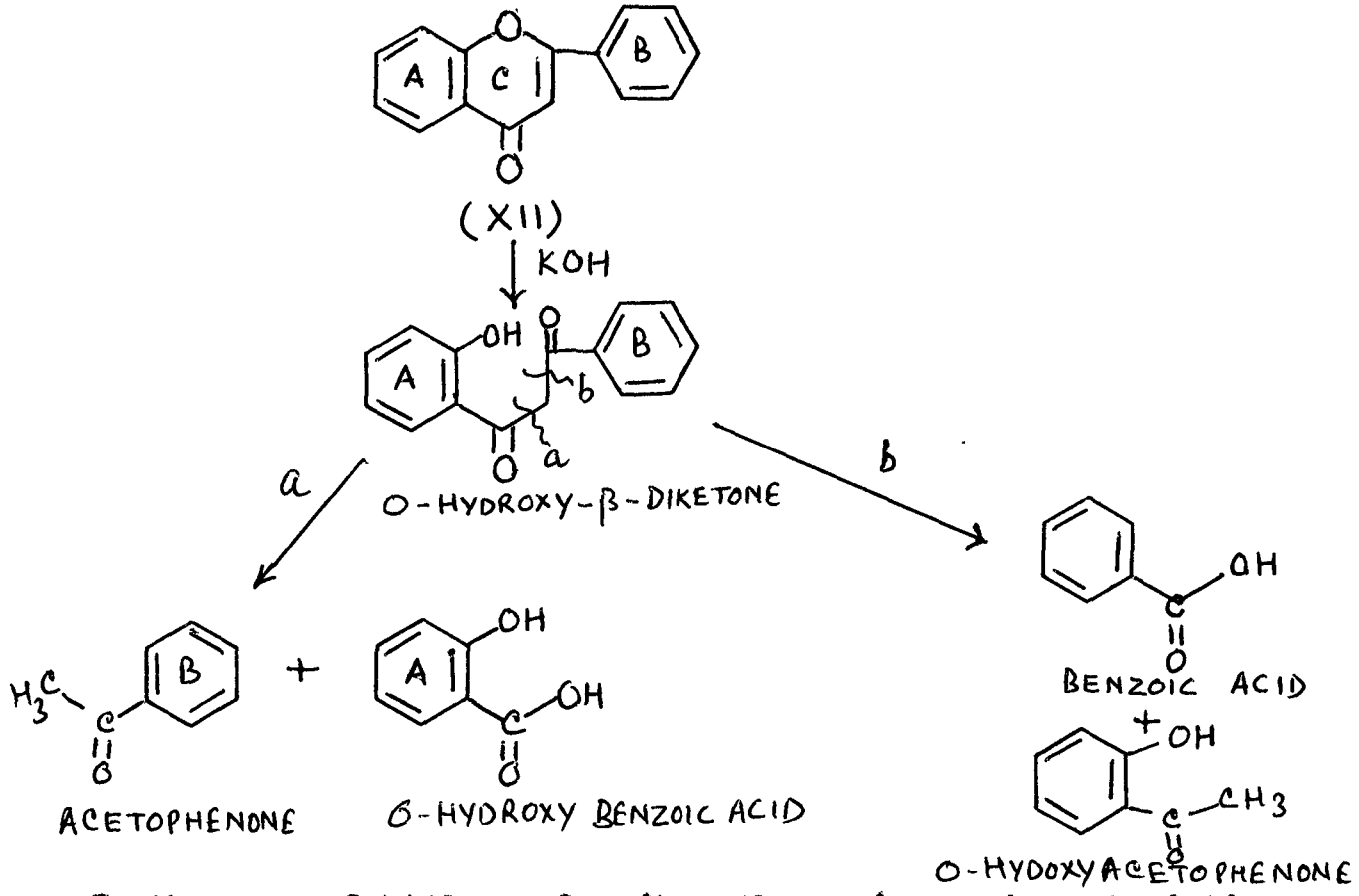
CHART XIV

3. DEGRADATION:

Degradation of biflavonyls can be brought about either by alkaline hydrolysis or oxidation with alkaline hydrogen peroxide.

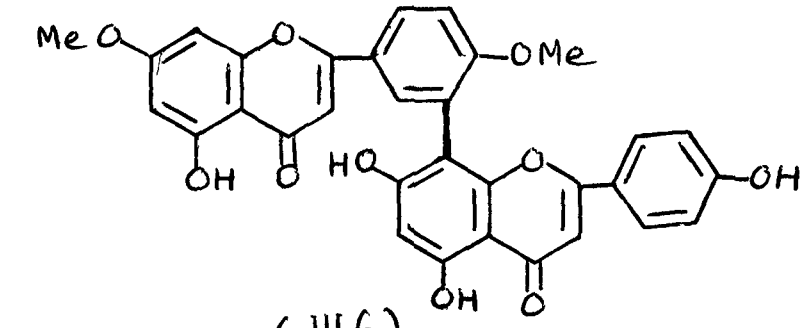
Alkaline Hydrolysis :

In general a flavone (XII) gives four products which arise by opening of the pyrone ring followed by the fission of the intermediate O-hydroxy-β-diketone by two different paths (a) and (b).

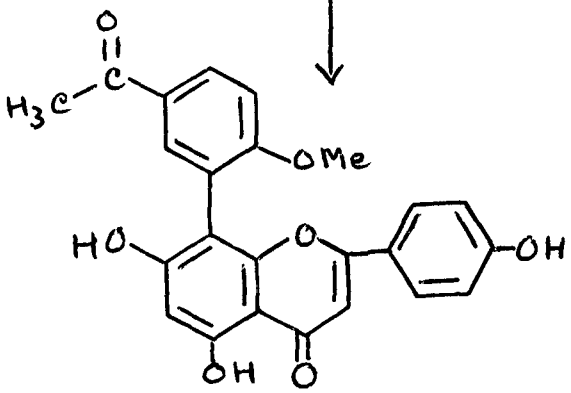


In the case of biflavonyls, 'ketoflavone' are characteristic degradation products of alkaline hydrolysis. Hydrolysis of ginkgetin (IIIf) by Kariyone and Kawano[135] gave a ketoflavone (LIII) containing

one methoxyl group, p-hydroxy acetophenone and 2,6-dihydroxy-4-methoxy acetophenone. IR spectrum of the diketone showed two carbonyl frequencies (1657 and 1645 cm^{-1}) and hydroxyl absorption. As the location of the methoxyl group at C-4' in ginkgetin is already established, the ketoflavone according to spectral evidence must have the structure (LIII). Alkaline hydrolysis of both

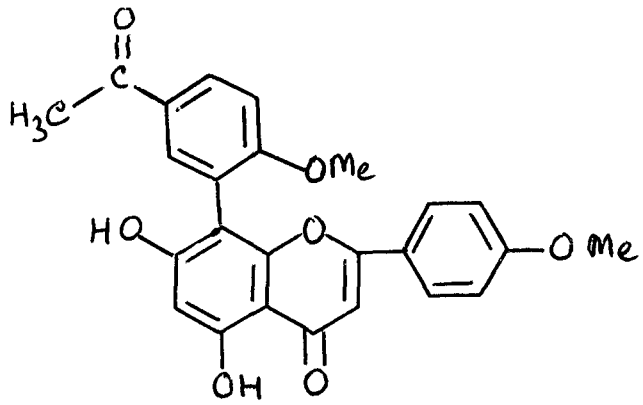


(IIIf)



(LIII)

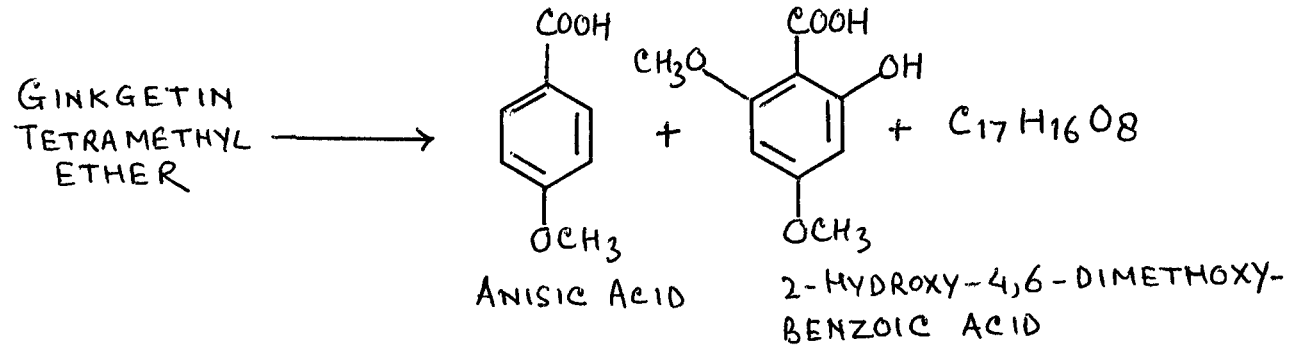
isoginkgetin (IIIg) and sciadopitysin (IIIj) gave the same ketoflavone (LIV) thus supporting the structure proposed for these two biflavonyls.[136]



(LIV)

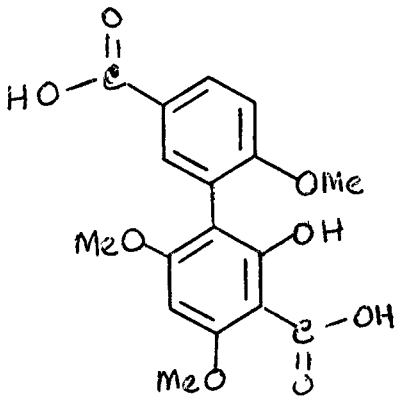
OXIDATION WITH ALKALINE HYDROGEN PEROXIDE:

Alkaline hydrogen peroxide oxidation has been very helpful in the determination of the interflavonyl linkage. Ginkgetin tetramethyl ether on oxidation with alkaline hydrogen peroxide, gave anisic acid, 2-hydroxy-4,6-dimethoxy benzoic acid and a compound ($C_{17}H_{16}O_8$).

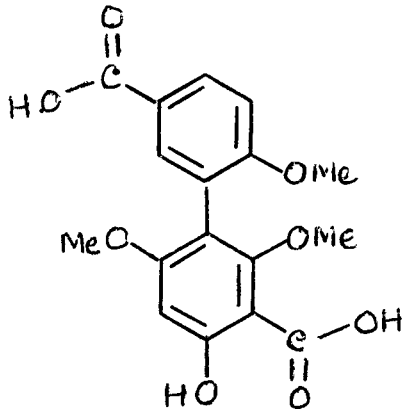


The compound ($C_{17}H_{16}O_8$) was shown to be a dicarboxylic acid, containing three methoxyl groups and one hydroxyl group. To fit

in all the spectral data, two structures A & B were postulated for the dicarboxylic acid.

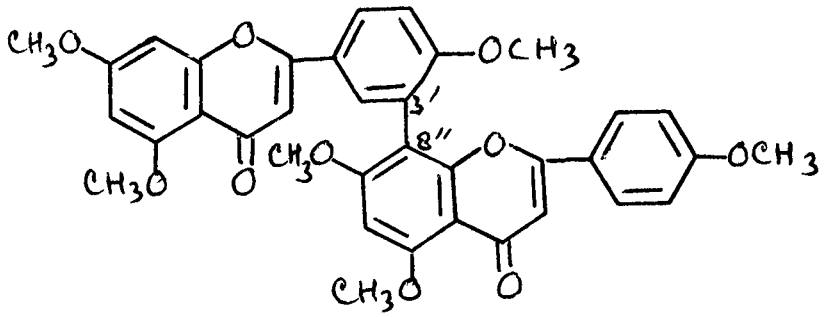


(A)

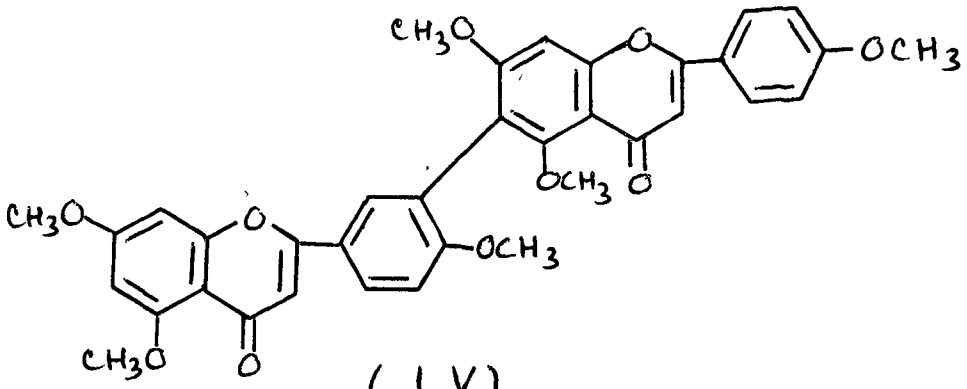


(B)

These facts proved that a biphenyl residue must exist in ginkgetin molecule and that interflavonyl linkage must involve position 3' of one flavonoid residue and 6 or 8 of the other. The two structures (XXXIII) and (LV) for ginkgetin tetramethyl ether were, therefore, considered.



(XXXIII)



(LV).

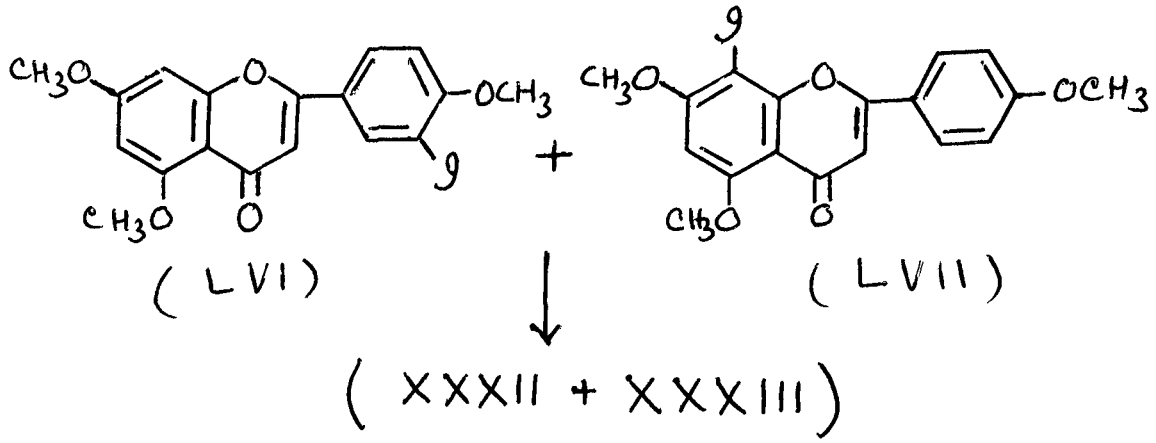
Of the two structures, the one involving C_3,-$C_{8''}$ linkage was preferred. The other structure (C_3,-$C_{6''}$) was considered unlikely since the 5''-OH in a compound with this structure would be severely hindered and there was no evidence that this hydroxyl group in ginkgetin was exceptionally difficult to methylate.[104] The structure with C_3,-$C_{8''}$ linkage was, therefore, proposed for ginkgetin tetramethyl ether (XXXIII) and structure (A) for the derived carboxylic acid.

4. SYNTHESIS :

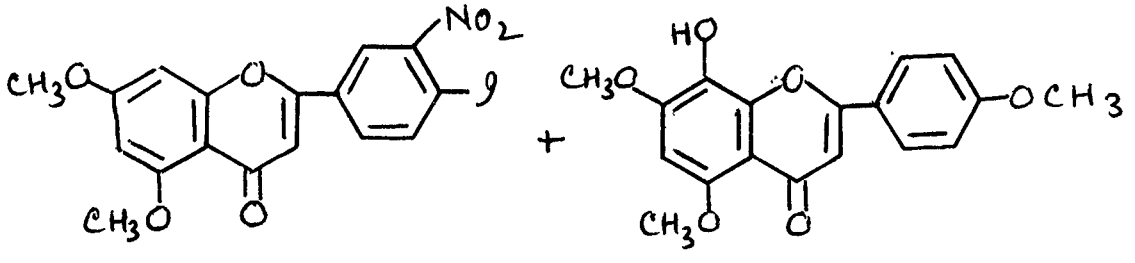
(A) ULLMANN COUPLING :

In 1955, Mahesh and Seshadri,[137] reported the first synthetic biflavonoid, as a byproduct of the oxidation of acetophenone with Fenton's reagent in acid medium by dehydrogenative coupling in the 3-position. Recently, a number of biflavonoids have been synthesised by application of Ullmann reaction.[138-141] Nakazawa

accomplished the synthesis of amentoflavone hexamethyl ether by mixed Ullmann reaction between 3'-iodo-4',5,7-tri-O-methyl flavone (LVI) and 8-iodo-4',5,7-tri-O-methyl flavone (LVII). Cupressuflavone hexamethyl ether was obtained as a byproduct and was found identical with the one obtained from natural sources. Later on Seshadri et al[30] have also synthesised cupressuflavone hexamethyl ether from 8-iodo-4',5,7-tri-O-methyl flavone (LVII) under modified conditions of Ullmann condensation.

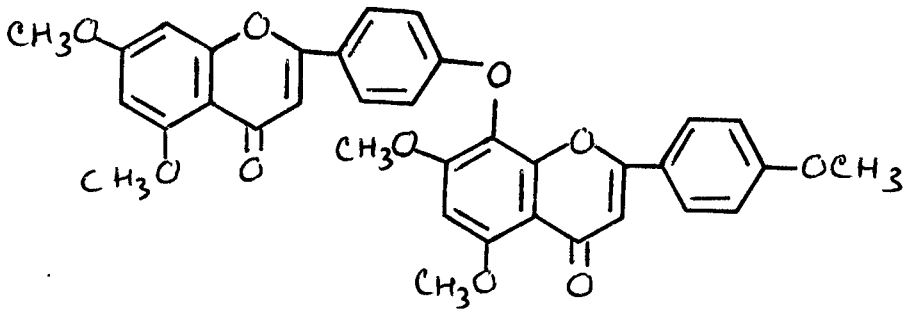


Recently 4'-O-8" and 4'-O-6" linked hinokiflavone have been synthesised by Nakazawa[7] in his elegant seventeen step synthesis. The permethylated 3'-nitrobiflavonyls, the key intermediates, were obtained by condensation of 3'-nitro-4'-iodo-5, 7-di-O-methylflavone (LVIII) and 8- and 6-hydroxy-4',5,7-tri-O-methyl flavones (LIX and LX) in DMSO in the presence of K_2CO_3. The nitro ethers were reduced by $Na_2S_2O_3$ in aqueous DMF, diazotized and decomposed with 50% H_3PO_2 to give pentamethyl ethers of hinokiflavones (XXXIV and XXXV).



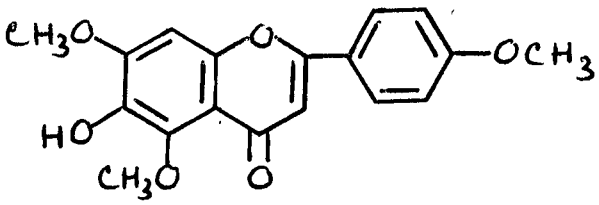
(LVIII)

(LIX)

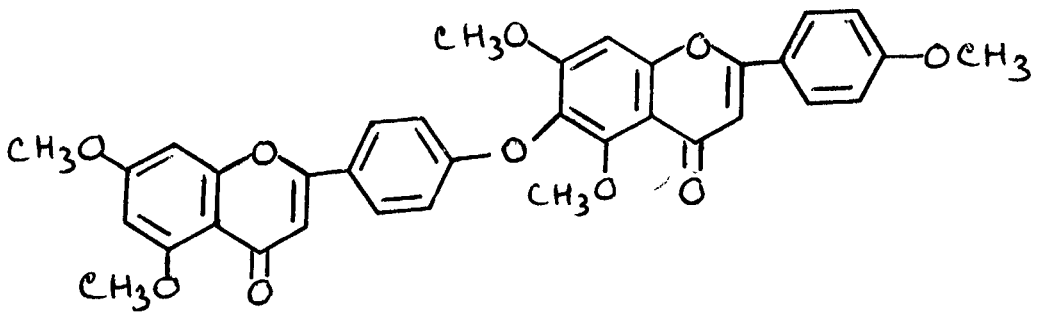


(XXXIV)

(LVIII) +



(LX)



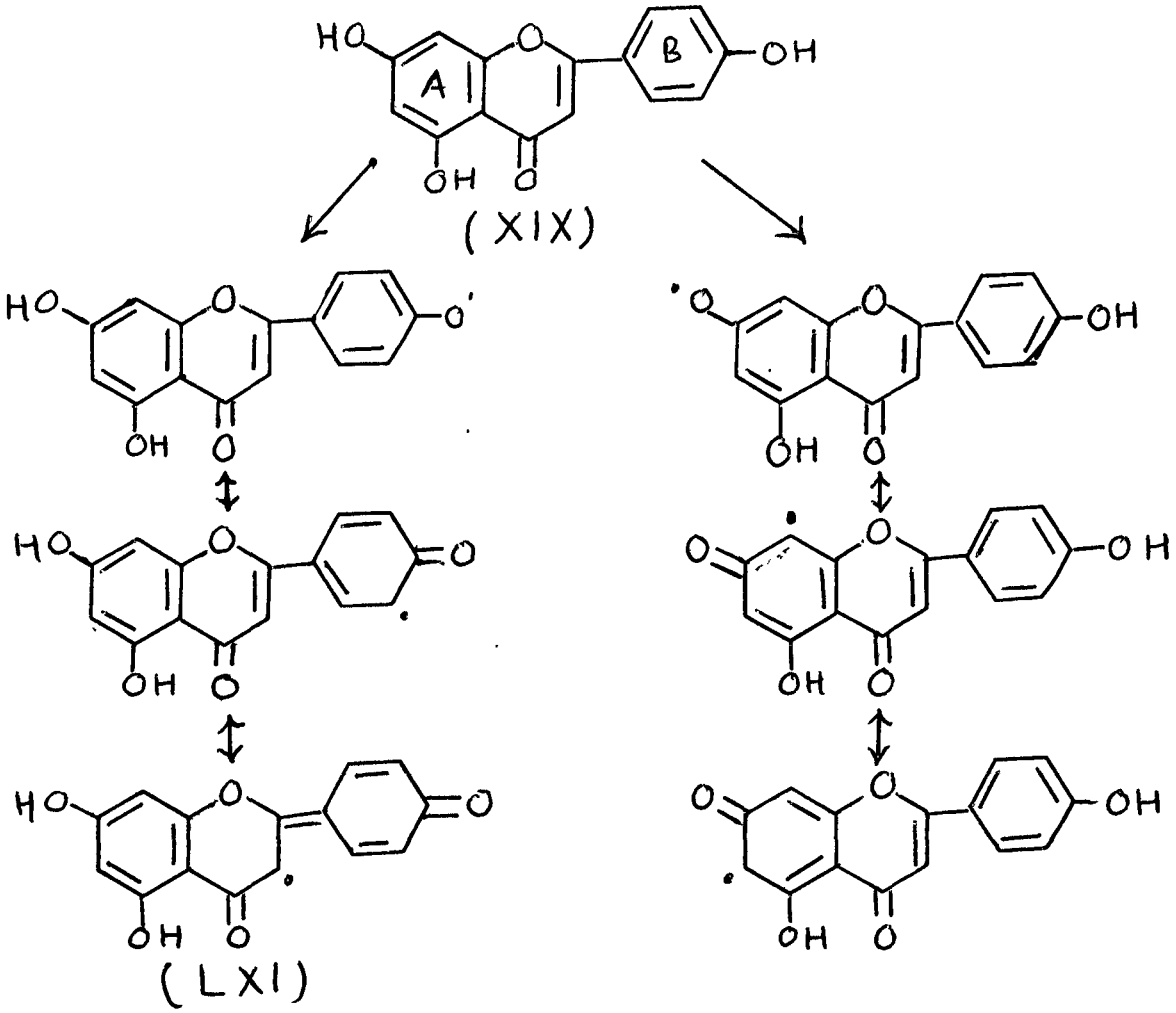
(XXXVI)

(B) OXIDATIVE COUPLING :

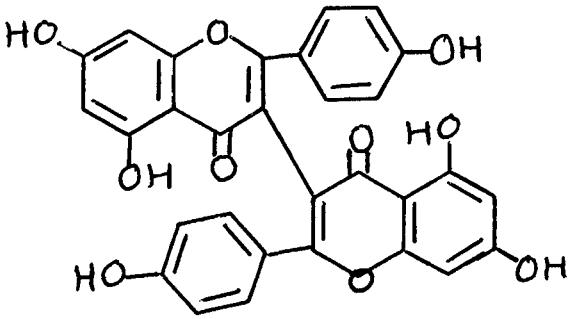
The oxidative coupling of free radical species derived from phenolic substrates is now widely accepted as the pathway by which many complex natural products are biosynthesized.[142,143] Such a route has been suggested as being involved in the formation of biflavonyls, which possess the apigenin moiety (XIX) as a common structural feature.[104] Thus the parent biflavonyls, amentoflavone (IIIa), cupressuflavone (Ia), agathisflavone (IIa) and hinokiflavone (VIIb) together with various O-methyl ethers, exhibit either C-C or C-O linked bonding between the flavonoid units which might be expected to arise through oxidative coupling of an apigenin-derived radical by three of the many modes of dimerization theoretically possible.

Oxidation of apigenin in aqueous sodium carbonate solution at room temperature under an atmosphere of nitrogen with potassium ferricyanide yielded two compounds with R_f values similar to those of amentoflavone and hinokiflavone (toluene-ethyl formate-formic acid, 5:4:1).[61] The structures of these dimers have been established as 3,3" and 3,3"'-biapigeninyl by spectral methods. The 3,3" and 3,3"' types of linkage, established for these two synthetic biflavonyls, do not correspond to the types of C-C bonding observed in those naturally occurring biflavonyls so far isolated.

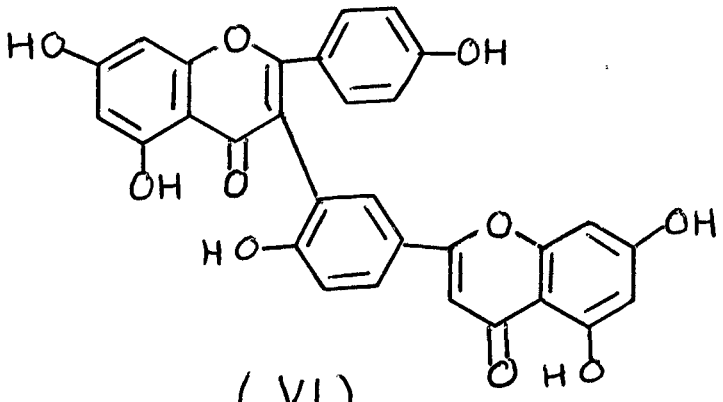
The synthetic compounds (V) and (VI) appear to arise by



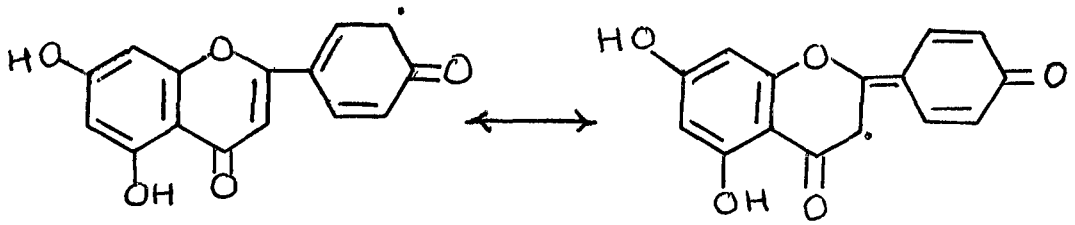
oxidative coupling of the radical (LXI), although none of the symmetrical 3',3"-linked dimer, which might also be expected to



(V)



(VI)



(LXI)

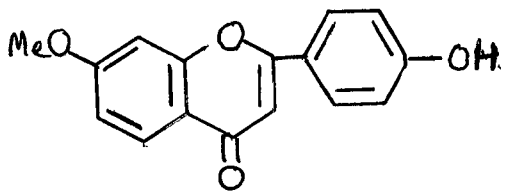
be formed, could be isolated. All three of the above modes of coupling have been observed in the laboratory with p-hydroxy styrenes and coniferyl alcohol, which may be regarded as the basic monomers for lignan and lignin biosynthesis, respectively.[142,143]

The recent report of isolation of 3,8"-flavonylflavanones[65,69] (VIIIa,b & g) and of 3,8"-biflavanonyls[75a] (IXa-d) shows that 3 position can be involved in the interflavonyl linkages of natural biflavonyls. It has been suggested that the biflavanonyls (VIIIa, b & c) are formed by oxidative coupling of cinnamic acid precursors, flavones or chalcones. However, the involvement of the latter in oxidative dimerization appears to conflict with the results of Dean and Podimuang,[144] who found that 2',4-dihydroxychalcones undergo intermolecular coupling on treatment with potassium ferricyanide to form 4'-hydroxyaurones in good yield.

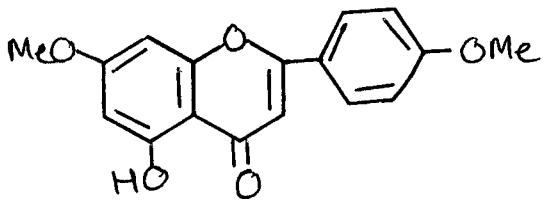
In contrast to the natural biflavonyls isolated todate, the synthetic dimers did not exhibit linkage through A ring. These results thus appear to indicate that the natural biflavonyls are formed not by radical coupling but rather by electrophilic attack

of the radical (LXI) on a second molecule of apigenin. Such a possibility has been suggested by Baker et al with substitution occurring at positions 6 or 8, since these would be the most susceptible to electrophilic attack.

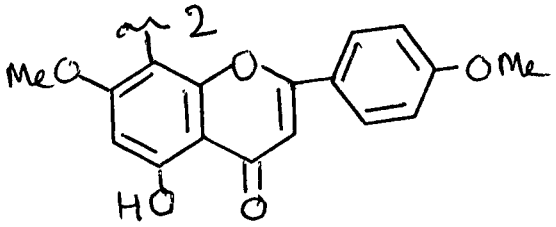
It is worthy of mention that Pelter et al[145] tried oxidative coupling of 4'-hydroxy-7-methoxyflavone (LXII) using potassium ferricyanide and got back the starting material. However, Seshadri et al[146] performed oxidative dimerisation of 5-hydroxy-4',7-di-O-methylapigenin (LXIII) with ferric chloride and claim to have isolated 8,8-biflavonyl (LXIV).



(LXII)



(LXIII)



(LXIV)

D I S C U S S I O N

BIFLAVONYLS OF THE ORDER ARAUCARIALES - THIN LAYER CHROMATOGRAPHIC IDENTIFICATION:

Earlier methods of detecting flavonoid pigments in plant extracts were based on simple colour tests, many of which were of doubtful validity, when used on crude extracts. Chromatographic methods have the advantages that they provide an accurate and rapid means of provisional identification based on R_f values and characteristic fluorescence in UV light. Further these methods can be applied to as little as 1 mg of unknown substances and many pigments have been identified without being isolated on a macro scale. Lastly the diversity of R_f values that can be obtained for each compound run in a variety of solvent mixtures provides a most useful aid to characterization.

Paper chromatography was first used for the separation of flavonoid pigments by Bate-Smith (1948).[82] Since then it has been used increasingly in the study of these compounds[83-85,147] which are idealy suited to this particular technique.

Suwada[148] and Hasegawa;[149] in their studies on taxonomic distribution of biflavonyls among gymnosperms have detected most of the biflavonyls by paper chromatography. De Modica et al;[150] during isolation of biflavonyls from Taxus baccata, observed that "with the classical solvent systems, the separations of biflavonyls, using both ascending and descending techniques on different chromatographic papers have not been satisfactory." Attempts of Baker

et al[29] to achieve quantitative separation of biflavonyls from various plant extracts by paper chromatography (Whatman No.3) also proved futile.

Thin-layer chromatography, the technique redeveloped by Stahl[159] in later fifties as a tool for quantitative separations, has many superiorities, such as speed, sensitivity, and efficiency of separation when compared to paper chromatography for the analysis of many types of compounds.

There have been some isolated reports as to the use of thin-layer chromatography for separation of biflavonyls from plant extracts[75a, 152]. Kawano et al[152] using this technique have revised many findings of Sawada[148] on the occurrence of biflavonyls in twelve gymnosperms.

Rahman et al[86] have given a systematic study on TLC of 38-biflavonyls and their derivatives, using five different solvent systems.

The present discussion is devoted to the systematic identification of biflavonyls from the leaf extracts of Araucaria bidwilli, A.cookii, A.cunninghamii, Agathis alba and A.palmerstonii using thin-layer chromatography.

The identification of parent biflavonyls and partial methyl ethers of the same series with the exception of isomeric mixtures, as found to occur in leaf extracts of Podocarpus gracillior[52] (Podocarpaceae) and Zamia floridina[46] (Cycadales) has already been reported.

The order Araucariales presents a special problem of detection and separation due to co-occurrence of complex mixtures of biflavonyls belonging to all the series known todate. It has, however, been possible to separate only mono- and dimethyl ethers of agathisflavone[32,33,38] and tetramethyl ethers of amentoflavone[57] and cupressuflavone[34] as single entities from such mixtures. The remaining constituents of the mixtures although structurally heterogeneous appeared chromatographically homogeneous and therefore undetectable and inseparable under the conditions used. The recent report of Natrajan et al[153] for the detection and separation of biflavonyl mixtures (Family-Cupressaceae) as such as well as their complete methyl ethers by TLC led us to use their procedure for the separation of biflavonyls of the order Araucariales.

The extract from the dried and powdered leaves (100 g) of each species after extraction with acetone and solvent fractionation followed by column chromatography was subjected to TLC examination. The chromatograms were run on silica gel G (E.Merck) using BPF (benzene-pyridine-formic acid, 36:9:5)[154] as developer. The complexities of all the chromatograms were revealed in U.V.light but the spots of biflavonyls and their partial methyl ethers were also located by using $FeCl_3$-EtOH and diazotized sulphanilic acid as chromogenic reagents.[155]

The two solvent systems (a) toluene:pyridine:acetic acid (10:1:1) and (20:1:1) (b) toluene-dimethylformamide-acetic acid

(10:1:1) accredited most satisfactory by the authors[153] were tried under the prescribed conditions. The system failed not only in separating the unresolved obstinate mixtures, but also those components which had earlier been easily and cleanly separated and fully authenticated. The same difficulty was encountered in detecting and separating them at the fully methylated stage. Two dimensional TLC in a number of solvent systems was also found to be unsatisfactory.

The complex mixtures of biflavonyls from the leaf extracts of Araucaria bidwillii, A.cookii, A.cunninghamii, Agathis alba and A.palmerstonii were examined. The chromatograms of the extracts of all the species are shown in Fig.VII. Fig.VIII shows the chromatogram of fully methylated product(s). The biflavonyl constituents identified in each species are recorded in Table-VI.

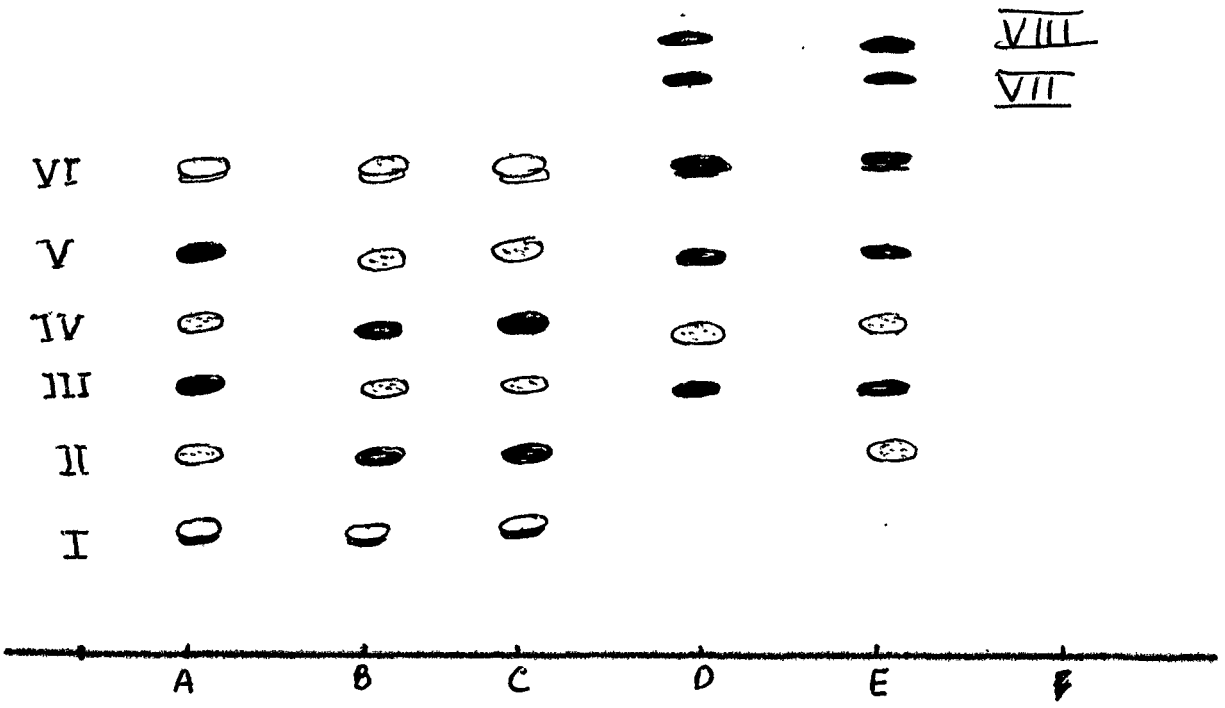


Fig.VII. Chromatogram in benzene-pyridine-formic acid (36:9:5) of biflavonyls from leaf extracts of :

(A) Araucaria bidwillii, Hooker,

(B) Agathis palmerstonii,

(C) Agathis alba, Foxworthy,

(D) Araucaria cookii R.Br.ex.D.Don

(E) Araucaria cunninghamii, Ait.

● - Major,

◎ - Medium,

○ - Minor.

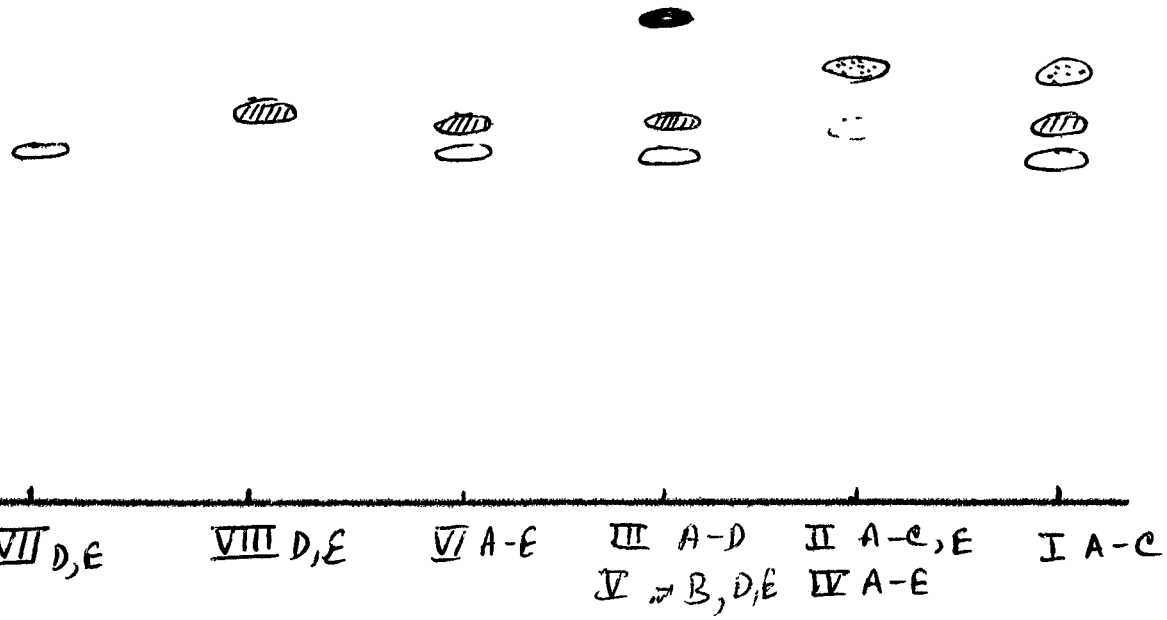
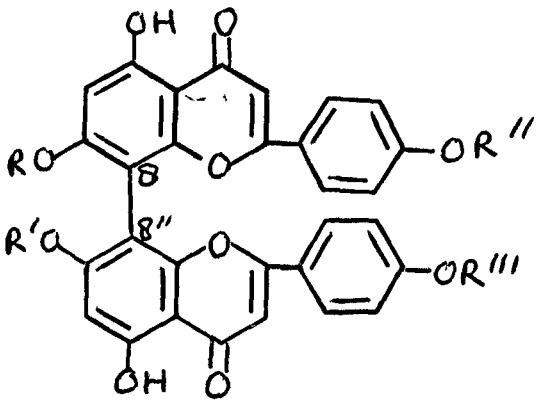


Fig.VIII. Chromatogram in benzene-pyridine-formic acid (36:9:5) of complete methyl ethers of band I to VIII from, (A) Araucaria bidwillii, Hooker, (B) Agathis palmerstonii, (C) Agathis alba, Foxworthy, (D) Araucaria cookii and (E) Araucaria cunninghamii, Ait.

- ⬭ - Amentoflavone hexamethyl ether (yellow),
- ⬭ - Cupressuflavone hexamethyl ether (orange),
- ⬭ - Agathisflavone hexamethyl ether (bright-yellow) and
- ⬬ - Hinokiflavone pentamethyl ether (blue).

Shades were noted in U.V.light.



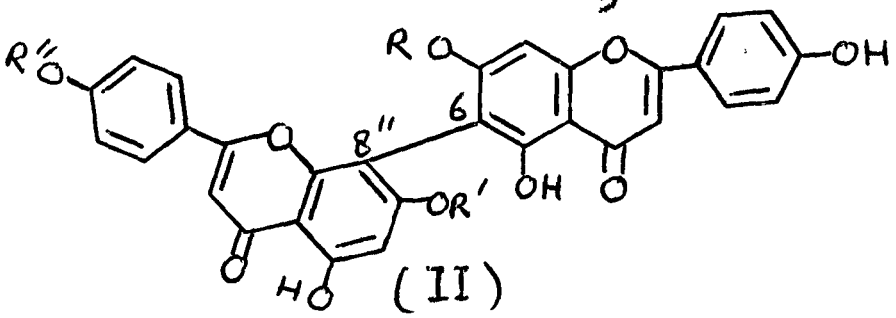
(I)

(b) R=CH_3; R'=R"=R"'=H

(c) R=R'=CH_3; R"=R"'=H

(d) R=R'=R"=CH_3; R"'=H

(e) R=R'=R"=R"'=CH_3



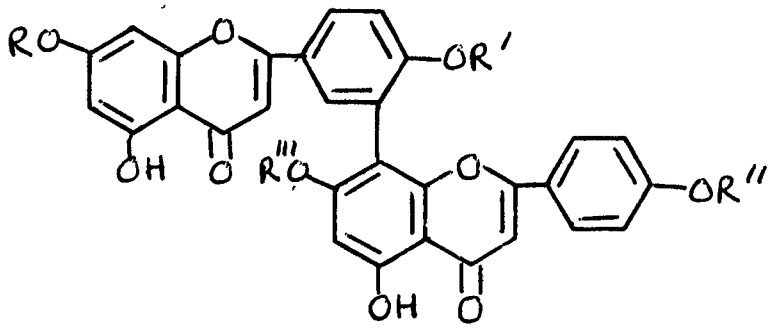
(II)

(a) R=R'=R"=H

(b) R=CH_3; R'=R"=H

(c) R=R'=CH_3; R"=H

(d) R=R"=CH_3; R'=H



(III)

(c) $R' = CH_3$; $R = R'' = R''' = H$

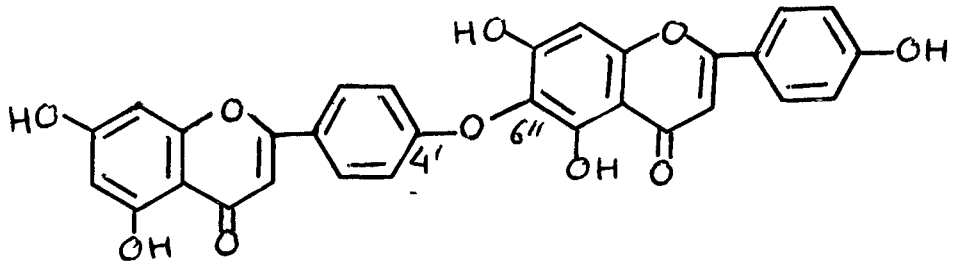
(d) $R''' = CH_3$; $R = R' = R'' = H$

(i) $R' = R''' = CH_3$; $R = R'' = H$

(j) $R = R' = R'' = CH_3$; $R''' = H$

(l) $R' = R'' = R''' = CH_3$; $R = H$

(m) $R = R' = R'' = R''' = CH_3$



(VII a)

TABLE-VI

DISTRIBUTION OF BIFLAVONYLS IN ARAUCARIALES

Band	R_f in BPF	Biflavonyls	A	B	C	D	E	Remarks
I	0.17	Ag	+	+	IIa			(IIa) Agathisflavone
		Cu	+	+	+			
		Am	+	+	+			
II	0.27	M-Ag	IIb	IIb	IIb		IIb	(IIb) 7-O-methylagathisflavone
		M-Cu	Ib	+	Ib	+	+	(Ib) 7-O-methylcupressuflavone
III	0.37	M-Am	IIIc	+	+	+	+	(IIIc) Bilobetin.
		Hi	+	+	+	VIIa		(VIIa) Hinokiflavone
IV	0.43	D-Ag	IIc	IId	+	IId	IId	(IIc) 7,7"-Di-O-methylagathisflavone (IId) 4"',7-Di-O-methylagathisflavone
		D-Cu	Ic	+	Ic	Ic	Ic	(Ic) 7,7"-Di-O-methylcupressuflavone
V	0.54	D-Am	+	+	+	IIIi	IIIi	(IIIi) 4',7"-Di-O-methylamentoflavone
		M-Hi		+		+	+	
VI	0.61	T-Cu	+	+	+	Id	Id	(Id) 4',7,7"-Tri-O-methylcupressuflavone
		T-Am	+	+	+	IIIj,IIIl	IIIl	(IIIj) Sciadopitysin (IIIl) Kayaflavone
VII	0.76	Te-Am				IIIm	IIIm	(IIIm) 4',4"',7,7"-Tetra-O-methylamentoflavone
VIII	0.77	Te-Cu				Ie	Ie	(Ie) 4',4"',7,7"-Tetra-O-methylcupressuflavone

A= Araucaria bidwillii; B= Agathis palmerstonii; C= Agathis alba; D= Araucaria cookii; E= Araucaria cunninghamii; Ag= Agathisflavone; Cu= Cupressuflavone; Am= Amentoflavone; Hi=Hinokiflavone; M= Mono- ; D=Di-; T=Tri- and Te=Tetra-methyl ethers. + = detected.

It is worthy of mention that not only closely spaced bands but also chromatographically homogeneous mixtures (including even minor constituents) such as (a) amentoflavone, cupressuflavone, agathisflavone (b) hinokiflavone, monomethyl ethers of amentoflavone and cupressuflavone (c) monomethyl ether of hinokiflavone, dimethyl ethers of amentoflavone and cupressuflavone and (d) trimethyl ethers of amentoflavone and cupressuflavone, after complete methylation are unequivocally detected by difference in R_f value and characteristic fluorescence in U.V.light (Fig.VIII) The fully methylated ethers,thus, may be separated by preparative TLC and authenticated although the separation or even detection of individual components of the original mixtures is not possible. Some of the constituents from such mixtures have, however,been obtained in pure form by CCD methods.[32,52]

The species examined show a novel feature of possessing biflavonyls belonging to all the series known so far. The presence of agathisflavone series seems to be characteristic of the order. Araucaria bidwillii, Agathis alba and Agathis palmerstonii are comparable in having biflavonyl mixtures from parent compounds to trimethyl ether while Araucaria cookii and Araucaria cunninghamii show the presence of biflavonyls from monomethyl-, to tetramethyl ethers. last but not the least important characteristic of the order is the optical activity as shown by some members.[33,34] These observations may prove useful to chemotaxonomists.

BIFLAVONYLS FROM ARAUCARIA BIDWILLI, HOOKER (ARAUCARIACEAE):

Leaves of Araucaria bidwilli, Hooker were procured from Horticulture Research Station, Saharanpur, U.P., India, and National Botanical Garden, Sibpore, West Bengal, India. Materials from both the places were investigated separately by identical procedures and found to contain the same biflavonyls.

The phenolic extractives of the coarsely powdered leaves by solvent fractionation, column chromatography (magnesium silicate) followed by preparative thin layer chromatography (silica gel G) yielded six components, four major and two minor. The components were labelled as WN_0, WN_1, WN_2, WN_3, WN_4 and WN_5. The usual colour reactions, ultraviolet spectra in ethanol and with various added diagnostic reagents indicated all of them to be flavonoids.

WN_0 and WN_5 were minor constituents and were detected by TLC examination and characteristic fluorescence in U.V.light as mixtures of (a) amentoflavone, cupressuflavone and agathisflavone and (b) trimethyl ethers of amentoflavone and cupressuflavone respectively. WN_1 and WN_3 were characterized as 7-O-methylagathisflavone and 7,7"-di-O-methyl agathisflavone respectively. The fraction WN_2, although homogeneous in chromatographic behaviour was separated into two components WN_{2a} and WN_{2b} by CCD between ethyl methyl ketone and borate buffer (pH 9.8) and characterised as bilobetin (IIIc) and 7-O-methyl-

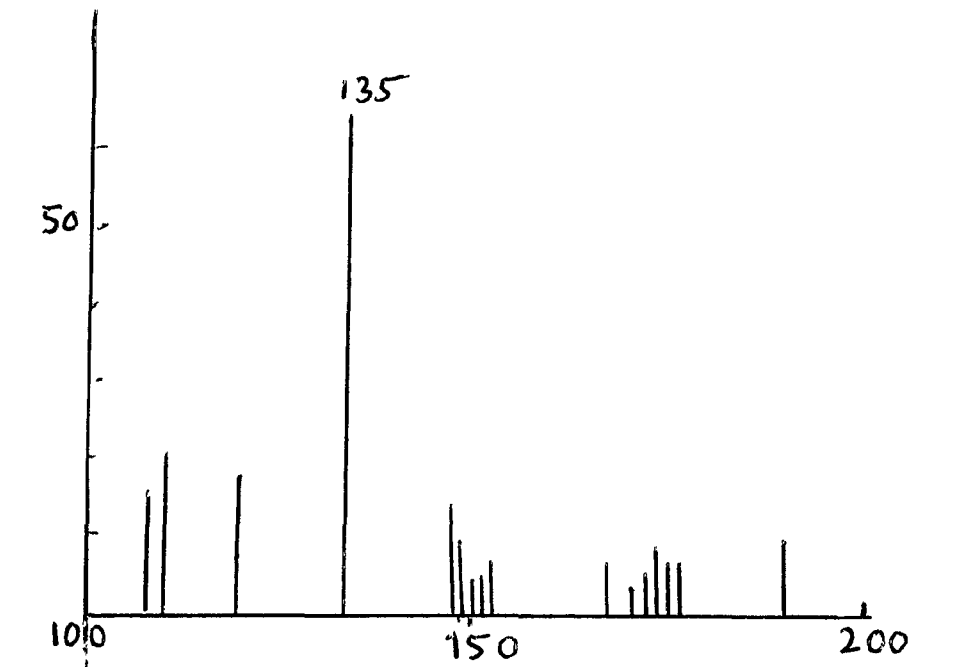
cupressuflavone (Ib) respectively. WN_4 was found to be a mixture of dimethyl ethers of amentoflavone and cupressuflavone by TLC examination of its fully methylated product. The methyl ethers WN_4M_1 and WN_4M_2 were separated and characterised as amentoflavone hexamethyl ether and cupressuflavone hexamethyl ether respectively. Acetylation of WN_4 followed by repeated crystallizations gave only biogenkwanin tetraacetate (WN_4A_1).

The structures of the different constituents have been fully elucidated by UV, IR, NMR and mass spectral studies.

4',4"',5,5",7-Pentahydroxy-7-O-methyl-6,8"-biflavonyl (WN_1) :

	m.p.	R_f	Mol.wt.
WN_1 (parent)	$<310^0$	0.27	552 (M^+)
WN_{1A} (acetate)	165-166°	-	762 (M^+)
$AgMe_6$ (methyl ether)	160-162°	0.45	622 (M^+)

The usual colour tests, TLC examination, ultraviolet spectra in ethanol and with various added diagnostic reagents indicated WN_1 to be a biflavonyl. Molecular weight determination (mass) of WN_1 established it to be a biflavonyl and that of WN_{1A} and $AgMe_6$ as its pentaacetate and pentamethyl ether respectively. $AgMe_6$, therefore, represents a biflavonyl hexamethyl ether.


135
50
100
150
200

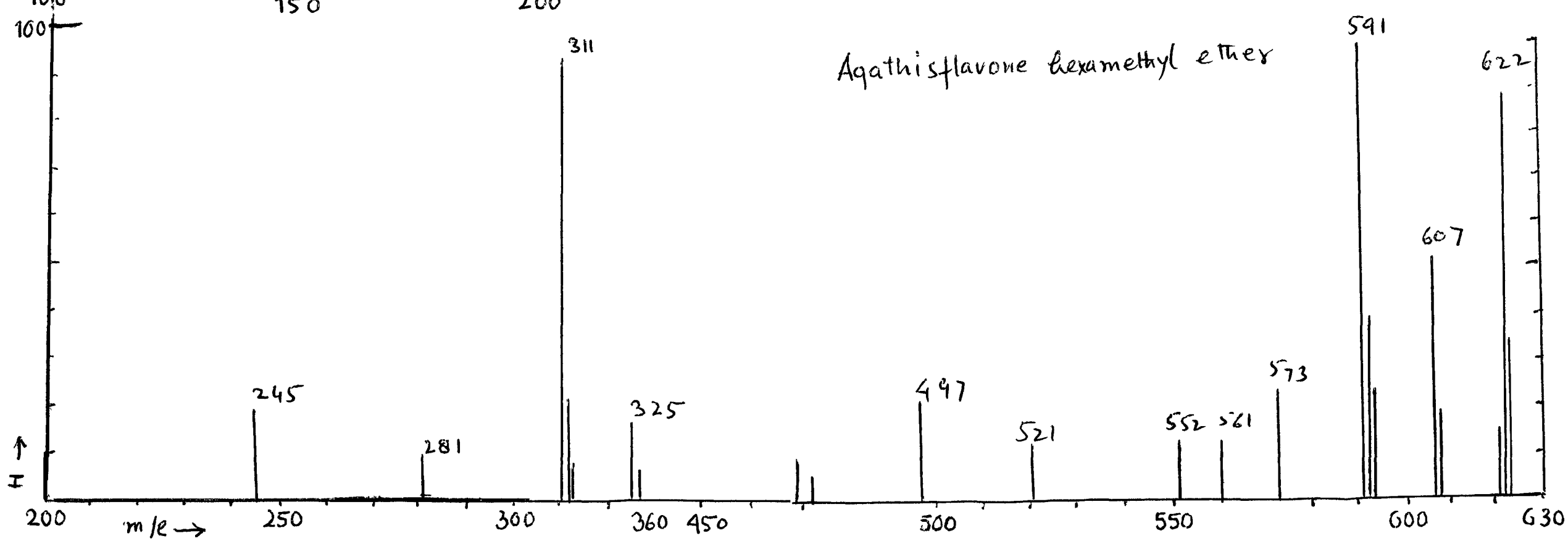

Agathisflavone hexamethyl ether
311
591
622
607
245
281
325
497
521
552
573
I
m/e →
200
250
300
360
450
500
550
600
630

Fig. IX

The mass spectrum of $AgMe_6$ is shown in Fig.IX. The mode of fragmentation is given in Chart-XV.

Main peaks:

622 (90); 607 (54); 591 (98); 573 (24); 561 (15); 521 (12); 497 (24); 325 (20); 311 (100); 281 (12); 245 (22) and 135 (65).

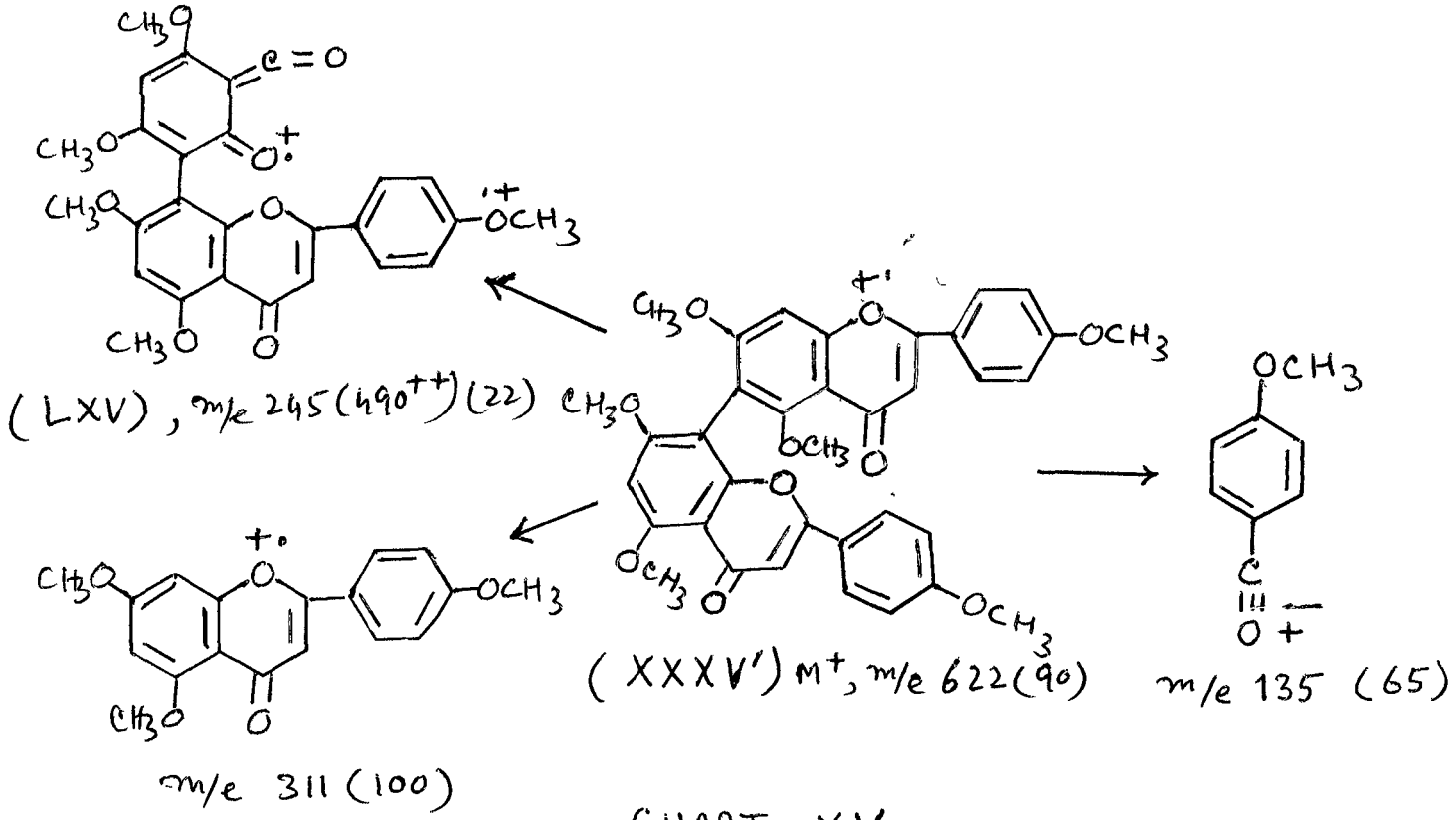


CHART - XV

The base peak appeared at m/e 311 and the molecular ion at m/e 622. The peak at m/e 311 indicates that there are three methoxy groups in each flavone portion of the molecule. A number of possible modes of retero Diels-Alder fragmentation of agathisflavone hexamethyl ether (XXXV) and of the apigenin trimethyl ether

units derived from it could be formulated. These ions could have the positive charge localized at any of the different oxygens. The important ion originating from such fissions was at m/e 135 (65). The ion at m/e 245 (22) was the doubly charged ion corresponding to (LXV).

The results of NMR studies of $AgMe_6$ are given in table-VII.

TABLE-VII

CHEMICAL SHIFTS OF PROTONS OF $AgMe_6$

Signal	Number of protons	J c/s	Assignment
2.12 (d)	2	9 c/s	H-2',6'
2.99 (d)	2	9 c/s	H-3',5'
2.63 (d)	2	9 c/s	H-2"',6"'
3.22 (d)	2	9 c/s	H-3"',5"'
3.09 (s)	1	-	H-8
3.36 (s)	1	-	H-6"
3.47, 3.49 (s)	1 each	-	[H-3 / H-3"]
6.41,6.14,6.26, 6.12, 6.22, 5.95 (s each)	18	-	OMe-4',4"', 7,7",5,5" respectively

s = singlet, d = doublet

Spectrum run in $CDCl_3$ at 100 MC; TMS as internal standard = τ 10.00.

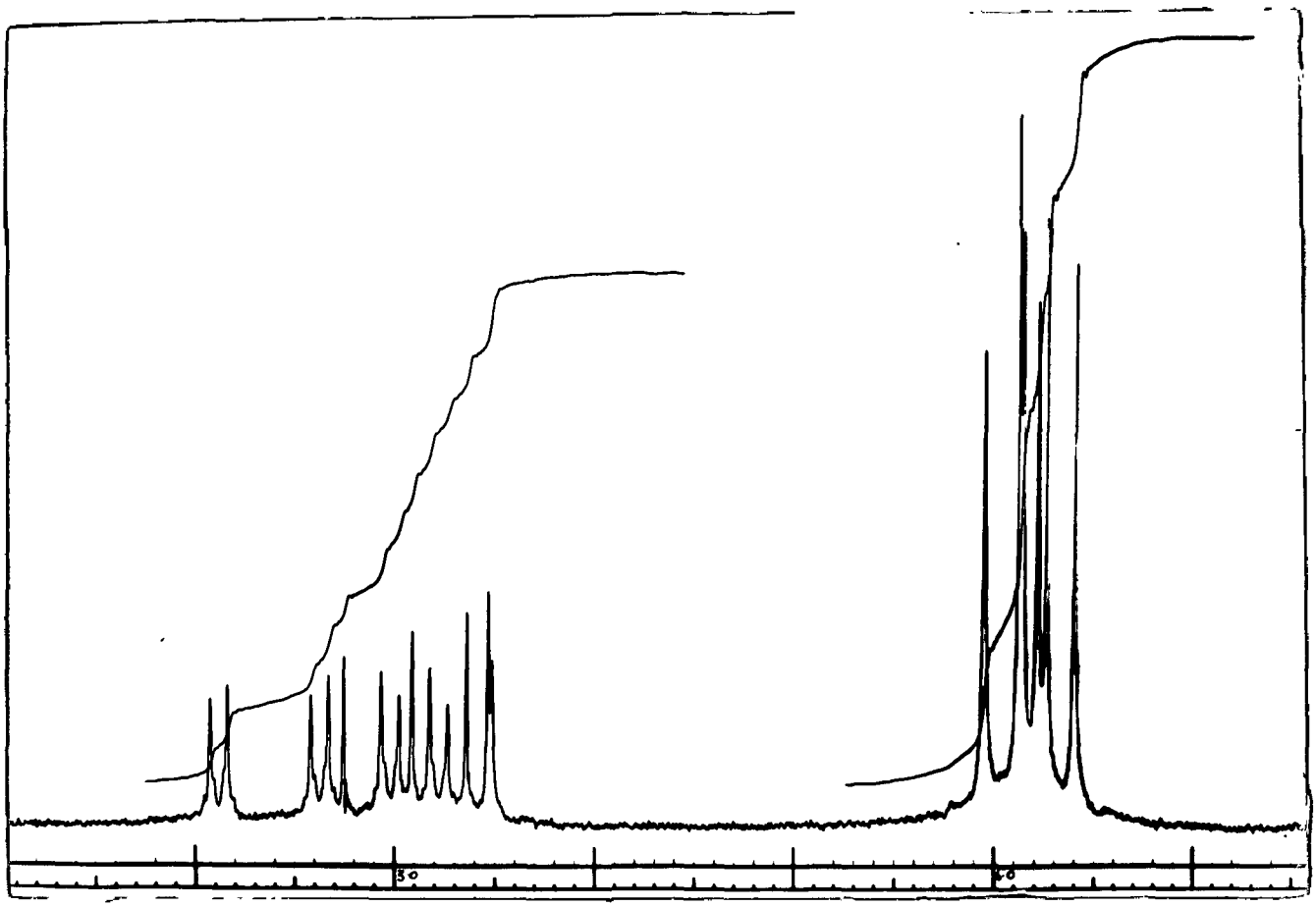


Fig. X

The NMR spectrum of $AgMe_6$ (Fig.X) showed that the molecule was neither symmetrical nor were the B and E rings implicated in linking the two flavonoid units as there were present two sets of A_2B_2 protons centred at τ 2.99, 2.12 (J=9 c/s) and τ 3.22 (J= 9 c/s), the pairing being proven by double irradiation experiments. J values were characteristic of ortho-coupled protons. The linkage could not be through C-3 or C-3" as there were almost two invariant protons at τ 3.40-3.50. Moreover this linkage would lead to at least one meta-coupled pair associated with rings A and D, and none, infact, was observed. This left only rings A and D implicated in the interflavonyl linkage, and as the compound was unsymmetrical (i.e. neither C_8-$C_{8"}$ nor C_6-$C_{6"}$) the linkage must be C_6-$C_{8"}$.

The observation that the 5-methoxy group of a 8-linked mono-flavonoid unit (attached to an aromatic ring of the biflavonyl appears below τ 6.00[62] is consistent with the proposed structure (Table-V).

TABLE-V

Biflavonoid	5-OMe	5"-OMe
1. Cupressuflavone hexamethyl ether	τ 5.85	τ 5.85
2. Amentoflavone hexamethyl ether	τ 6.13	τ 5.94
3. Agathisflavone hexamethyl ether	τ 6.41	τ 5.95
4. Hinokiflavone pentamethyl ether (C_4'-O-$C_{8"}$)	τ 6.00	τ 5.92
5. $AgMe_6$	τ 6.41	τ 5.95

The mode of interflavonyl linkage (C_6-$C_{8''}$) was further confirmed by studying the solvent induced shifts of methoxy resonances. On change of solvent from deuterochloroform to benzene, five methoxy groups showed large upfield shifts. One methoxy group was unique in that upto $\simeq$50% dilution with benzene no shift was seen and then a strong downfield shift was evidenced (Fig.II and Table-IV), a phenomenon seen in neither the amentoflavone nor cupressuflavone series. It was reasonable to assume that the methoxy group in question was the one at C-5, flanked by ring D on one side and a carbonyl group on the other.

TABLE-IV

SHIFT OF METHOXY RESONANCES OF A Me_6

Signal in $CDCl_3$ c/s	Signals in C_6H_6 c/s	Shifts in c/s
405	358	47 upfield
390	330	60 "
389	335	54 "
380	326	54 "
375	305	70 "
362	385	23 downfield

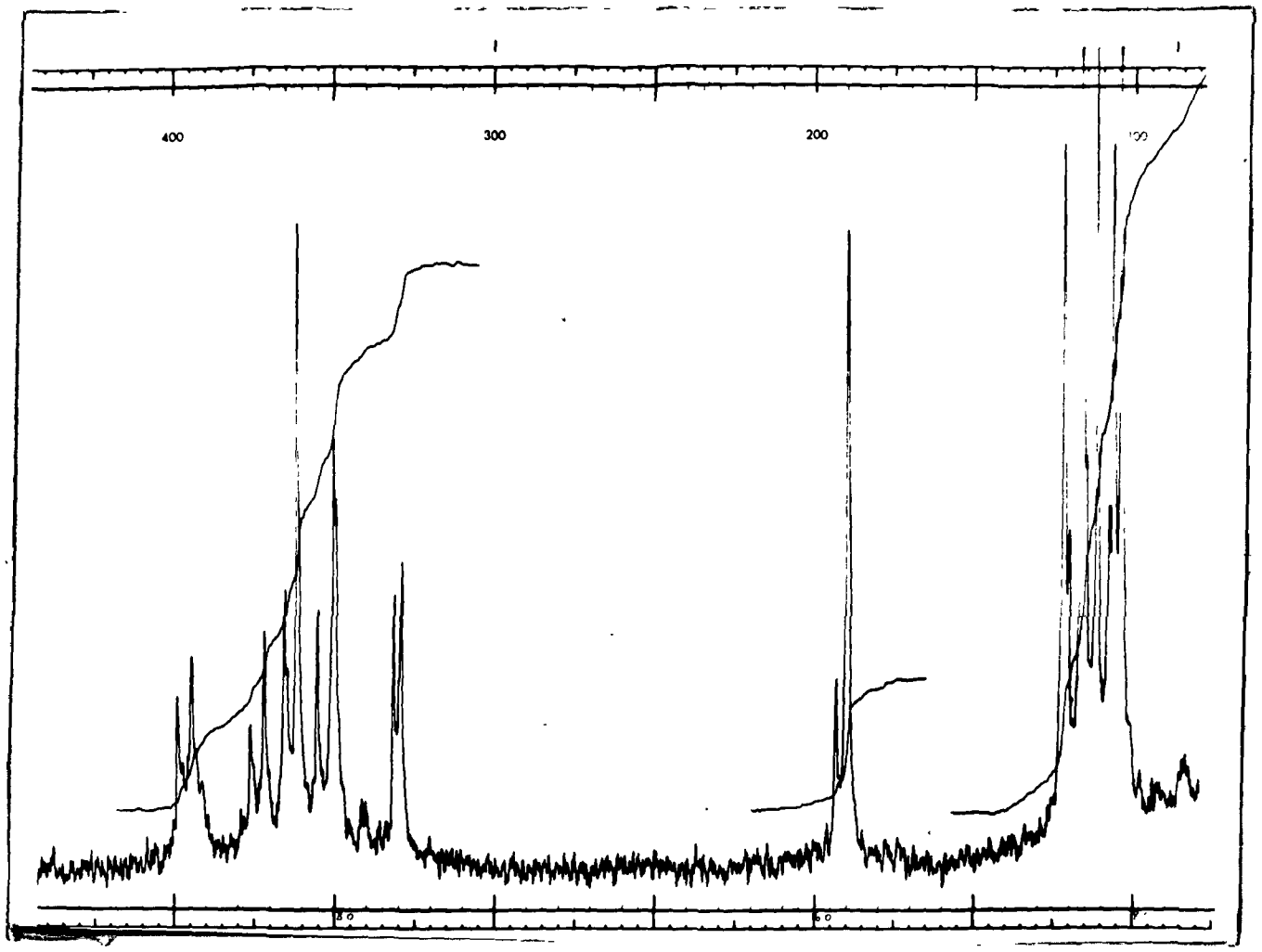


Fig - XI

The NMR spectrum of WN_{1A} is shown in Fig.XI. The results of NMR studies of WN_1 and WN_{1A} are given in Table-VIII and Table-IX respectively.

TABLE-VIII

CHEMICAL SHIFTS OF PROTONS OF WN_1

Signals	No. of protons	J c/s	Assignment
2.04 (d)	2	9	H-2',6'
2.95 (d)	2	9	H-3',5'
2.41 (d)	2	9	H-2"',6"'
3.88 (d)	2	9	H-3"',5"'
3.16 (s)	1	-	H-8
3.28 (s)	1	-	H-6"
3.40, 3.61 (s)	2	-	H-3,3"
6.13	3	-	OMe-7
0.8-1.2	3	-	OH-4',4"',7"
-3.07,-3.3	2	-	OH-5,5"

s = singlet, d = doublet

Spectrum run in $(CD_3)_2CO$ at 100 Mc. TMS as an internal standard $=\tau 10.00$.

TABLE-IX

CHEMICAL SHIFTS OF PROTONS OF WL-1A

Signals	No.of protons	J c/s	Assignment
2.08 (d)	2	9	H-2',6'
2.62 (d)	2	9	H-3',5'
2.50 (d)	2	9	H-2"',6"'
2.94 (d)	2	9	H-3"',5"'
3.00 (s)	1	-	H-8
3.01 (s)	1	-	H-6"
3.38, 3.42 (s)	2	-	H-3,3"
6.20 (s)	3	-	OCH_3-7
7.56, 7.66, 7.76, 7.86, 7.91 (s each)	15	-	OAc-5",5,4"', 4',7" respectively

s = singlet, d = doublet

Spectrum run in $CDCl_3$ at 100 Mc; TMS as internal standard=τ10.00.

The signal at τ3.36 (Table-VII) was assigned to H-6" in analogy with the chemical shifts of such protons on an 8-linked

flavone ring:

		H-6"
(a)	4',4"',5,5",7,7"-Hexa-O-methyl cupressuflavone	τ 3.44
(b)	4',4"',7,7"-Tetra-O-methyl-5,5"-dihydroxy-cupressuflavone	τ 3.42
(c)	4',4"',5,5",7,7"-Hexa-O-methylamentoflavone	τ 3.41

The proton at τ 3.09 (Table-VII) was assigned to H-6 of ring A in accordance with the observation that H-8 of 5,7-dimethoxyflavone is τ 0.20 downfield than H-6. The high position of H-6" (τ 3.36) as compared to H-8 (τ 3.09) was also in conformity with its location between oxygen atoms of two methoxyls at C-5" and C-7". The magnitude of downfield shifts of these protons on acetylation (Table-IX) was also important. Kassicot et al[102] have shown that acetylation of 5-hydroxy group in flavone moves the H-6, downfield by τ 0.22-0.29 and H-8 by τ 0.33-0.49, i.e. the extent of downfield shifts is greater for H-8. In the present case the greater downfield shift (τ 3.36-3.01=τ 0.35) of H-6" as compared to that of H-8 (τ 3.09-3.00 = τ 0.09) might be attributed to the presence of two acetoxy groups adjacent to H-6" in ring D.

The protons assigned to H-2"',6"' (τ 2.63) and H-3"',5"' (τ 3.22) positions of ring E fit well by analogy with the following similarly constituted E rings of 8-linked flavonoid units.

	2"',6"'	3"',5"'
(a) 4',4"',5,5",7,7"-Hexa-O-methyl-cupressuflavone	τ 2.62	τ 3.20
(b) 4',4"',7,7"-Tetra-O-methyl-5,5"-dihydroxy -	τ 2.57	τ 3.16
(c) 4',4"',5,5",7,7"-Hexa-O-methyl-amentoflavone	τ 2.68	τ 3.28
(d) 4',4"',7,7"-Tetra-O-methyl-5,5"-dihydroxy-	τ 2.56	τ 3.20

All these have an 8-linked flavone unit so that the protons at τ2.12 and τ2.99 were assigned to 2',6' and 3',5' positions respectively of the ring B of the flavonoid unit linked by the hitherto unknown 6-position. It was not possible to distinguish between C-3 and C-3" protons (Tables -VII-IX).

B_2 of both A_2B_2 pairs (rings B & E) in acetate moved downfield as compared to the methyl ether as well as the parent compound. This confirmed the presence of 4'-OAc and 4"'-OAc. Further, in parent compound itself (Table-VIII) there were certainly two hydrogen bonded hydroxy groups at τ-3.07 and τ3.30 as would be expected for C-5 and C-5" hydroxy groups. The methoxy group was not located at C-4' or C-4"' as shown earlier. These considerations left the only possibility of assigning the methoxy group to either C-7 or C-7". It was placed at C-7 due to the following considerations:-

(a) One singlet proton at τ3.36 in methyl ether ($AgMe_6$) moved to τ3.01 in acetate (WN_{1A}) showing the presence of two acetoxy groups in ring D. This compares well, with H-6" of sciadopitysin triacetate at τ3.04.

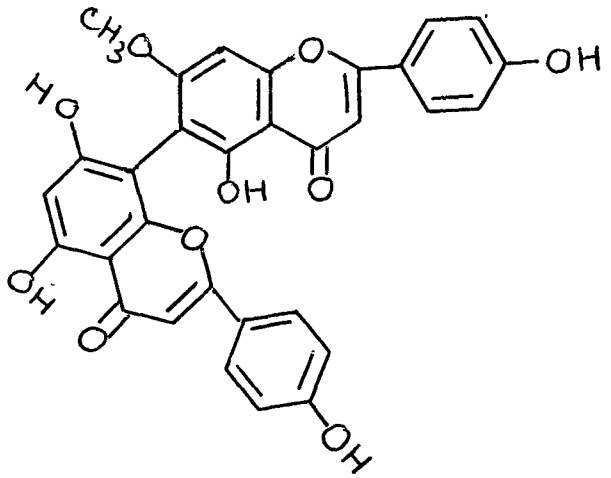
(b) H-6 of 5,7-diacetoxyflavone appears at τ3.15, while H-8 at τ2.45.

Further, confirmation that the single methoxy group was at C-7 comes in considering the situationif it were placed at C-7". The proton at C-6" in WN_1-acetate would be expected at τ 3.20 as shown under:

Diacetyl-4',4"',7,7"-tetra-O-methylcupressuflavone	τ 3.22
Tetraacetyl-7,7"-di-O-methylcupressuflavone	τ 3.19
Diacetyl-4',4"',7,7"-tetra-O-methylamentoflavone	τ 3.27

No signal was in fact seen in this region.

The compound WN_1 was, thus, assigned the structure 4', 4"',5,5",7"-pentahydroxy-7-O-methyl-6,8"-biflavonyl (IIb)



(IIb)

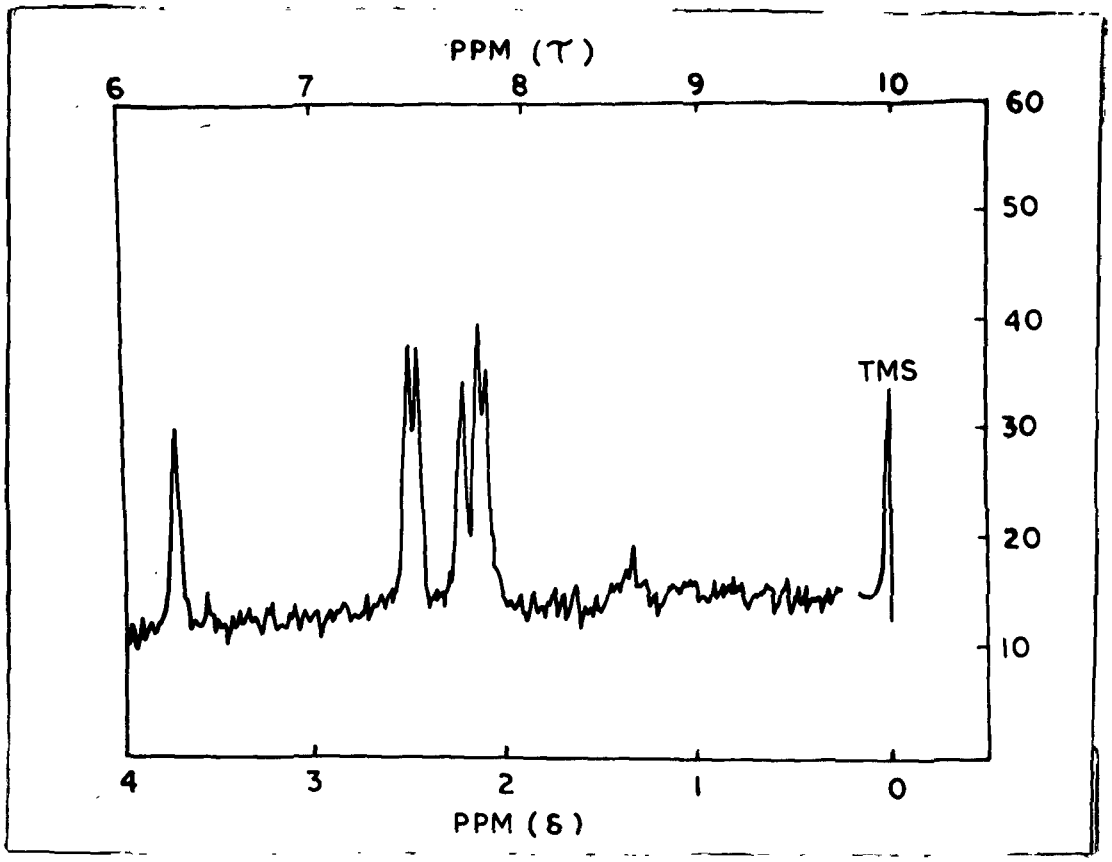


Fig. XII

4"',5,5",7,7"-Pentahydroxy-4'-O-methyl-3',8"-biflavonyl (WN_{2a}) :

WN_{2a} (parent)	m.p.	$<300^{\circ}$,	R_f 0.37,	Mol.wt. 552 (M^+)
WN_{2aA} (acetate)	m.p.	183-84°	-	Mol.wt. 762 (M^+)

TLC examination of the parent compound and its complete methyl ether and mass spectrum of WN_{2a} acetate m/e 762 (M^+) showed it to be a monomethoxypentaacetate of amentoflavone. The λ_{max}^{EtOH} appeared at 275 mμ(Band I) and 332 mμ(Band II). Addition of N/50 NaOEt caused a bathochromic shift of band I with an increase in intensity and of band II with a moderate decrease in intensity thus indicating that no methoxy group was present at C-7 or C-7".

The structure of WN_{2a} was further elucidated by comparison of methoxy and acetoxy resonances of WN_{2a} acetate with those of authentic samples (Table-X). The NMR spectrum of WN_{2a}-acetate is given in Fig.XII.

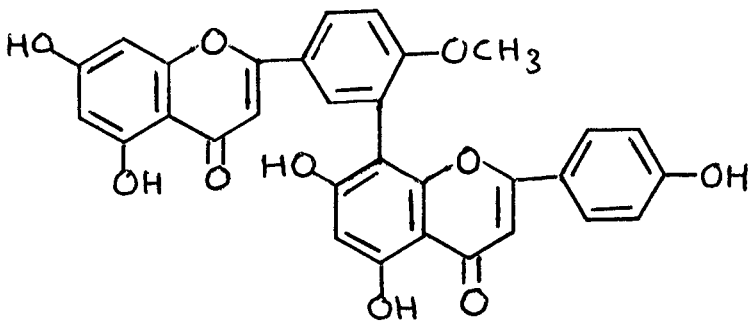
TABLE-X

CHEMICAL SHIFTS (τ SCALE) OF METHYL AND ACETYL PROTONS IN PYRIDINE SOLUTION

Compound	Assigned position in Biflavonyl Nucleus					
	4'	4"'	5	5"	7	7"
WN_{2a}-acetate	(6.28)	7.87	7.54	7.47	7.78	7.91
Bilobetin penta-acetate	(6.28)	7.85	7.53	7.49	7.76	7.90
Podocarpusflavone A pentaacetate	7.95	(6.44)	7.56	7.48	7.78	7.84
Sequoiaflavone pentaacetate	7.95	7.85	7.53	7.49	(6.28)	7.90
7"-O-Methylamentoflavone pentaacetate	7.95	7.85	7.55	7.48	7.77	(6.18)

Numbers in parentheses show the chemical shifts of methoxy protons.

The NMR of WN_{2a}-acetate was found to be identical with that of bilobetin pentaacetate. WN_{2a} was, therefore, assigned the structure 4'-O-methylamentoflavone (Bilobetin, IIIc).



(IIIc)

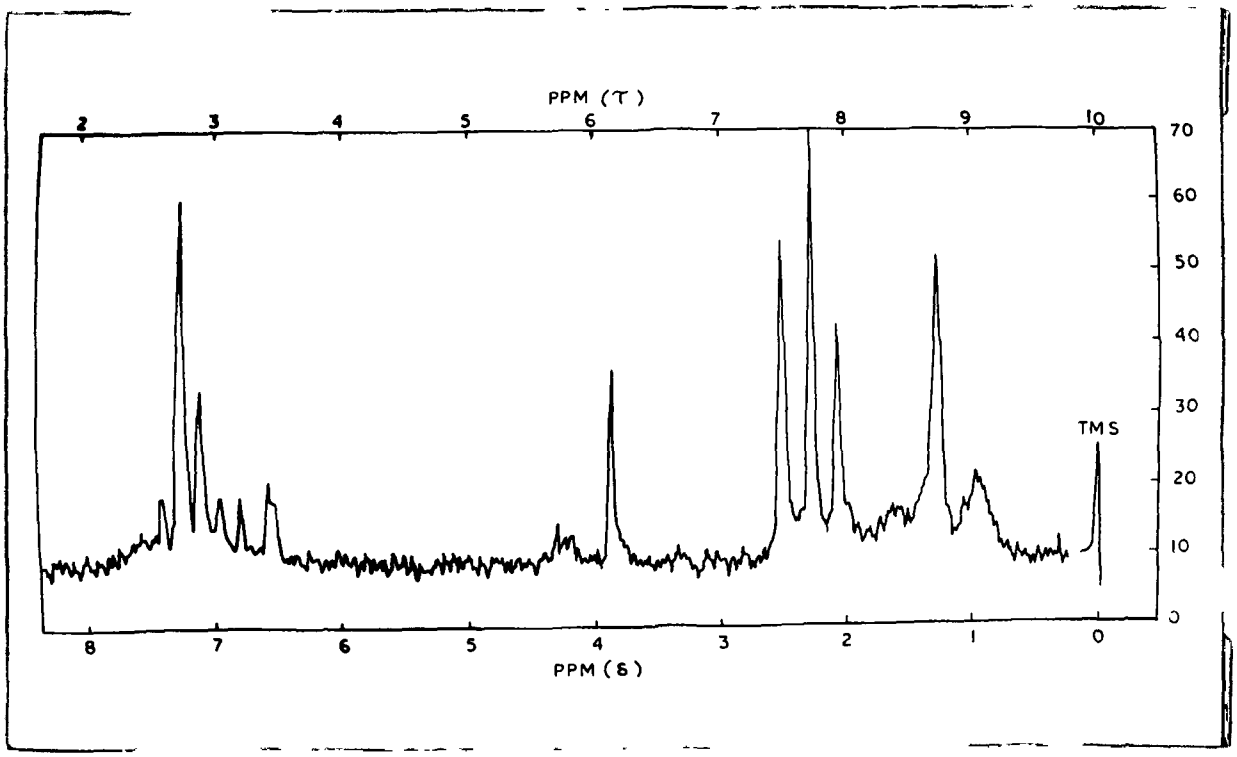


Fig. XIII

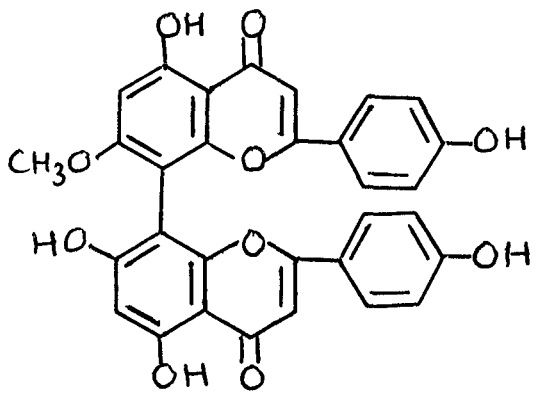
4',4"',5,5",7"-Pentahydroxy-7-O-methyl-8,8"-biflavonyl (WN_{2b}) :

	m.p.	R_f	Mol.wt.
WN_{2b} (parent)	186-90°	0.37	552 (M^+)
WN_{2bA} (acetate)	147-50°	-	762 (M^+)

TLC examination of WN_{2b} and its methyl ether and mass spectrum of its acetate (m/e 762,M^+) indicated that WN_{2b} might be monomethyl derivative of cupressuflavone.

The NMR spectrum of WN_{2bA} (Fig.XIII, Table-XI) showed two 4H doublet at τ2.97 (J = 9 c/s) and τ2.67 (J= 9 c/s) for H-3',5',3"',5"' and H-2',6',2"',6"' respectively, suggesting no difference between 4' and 4"' substitution groups. The two sets of singlets at τ3.49, τ3.44 and τ3.21, τ2.91 were assigned to H-3 (3") and H-6(6") respectively by comparison with the chemical shifts of cupressuflavone hexaacetate.

By comparison of NMR values of WN_{2bA} with those of cupressuflavone hexaacetate, WN_{2b} was assigned the structure 4',4"', 5,5",7"-pentahydroxy-7-O-methyl-8,8"-biflavonyl (Ib).



(I b)

TABLE-XI

CHEMICAL SHIFTS OF PROTONS

Assigned positions	WN_{2bA}	Cupressuflavone hexaacetate
H-3 (3")	3.49 (H,s) 3.44 (H,s)	3.44 (2H,s)
H-6 (6")	3.21 (H,s) 2.91 (H,s)	2.94 (2H,s)
H-3',5'(3"',5"')	2.97 (4H,d) J= 9 c/s	2.99 (4H,d) J= 9 c/s
H-2',6'(2"',6"')	2.67 (4H,d) J=9 c/s	2.71 (4H,d) J=9 c/s
4'(4"')	7.73 (6H,s)	7.75 (6H,s)
7(7")	6.15 (3H,s) 7.94 (3H,s)	7.92 (6H,s)
5(5")	7.50 (6H,s)	7.52 (6H,s)

s = singlet, d = doublet.

Spectrum run in $CDCl_3$ on a Hitachi H-60 instrument;
TMS as internal standard = τ 10.00.

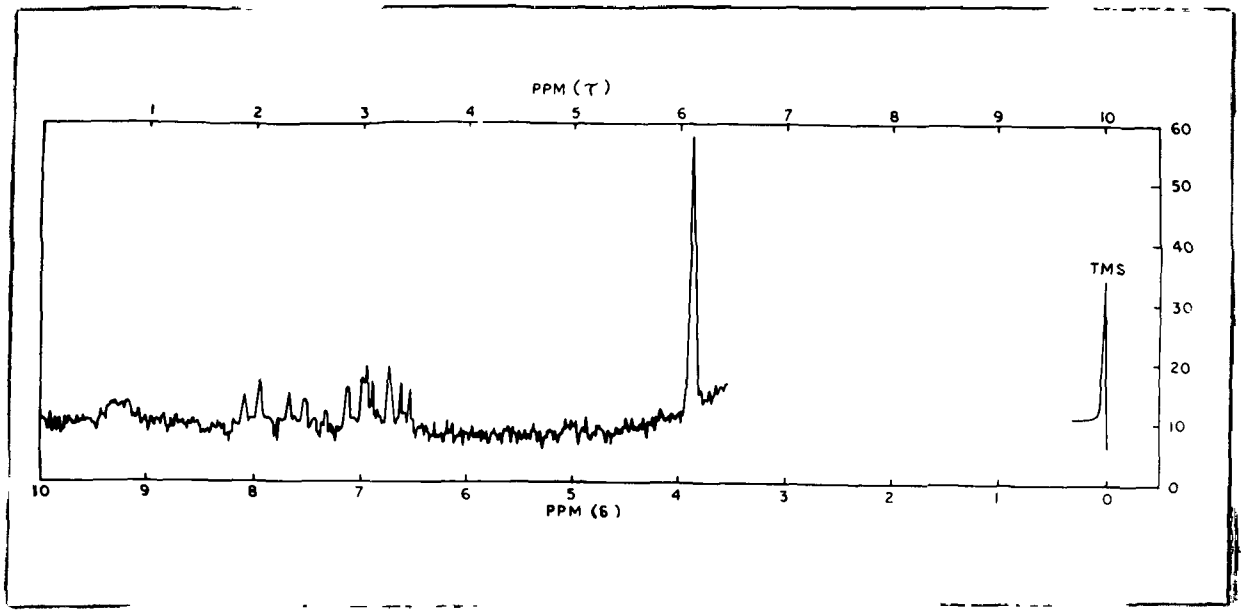


Fig. XIV

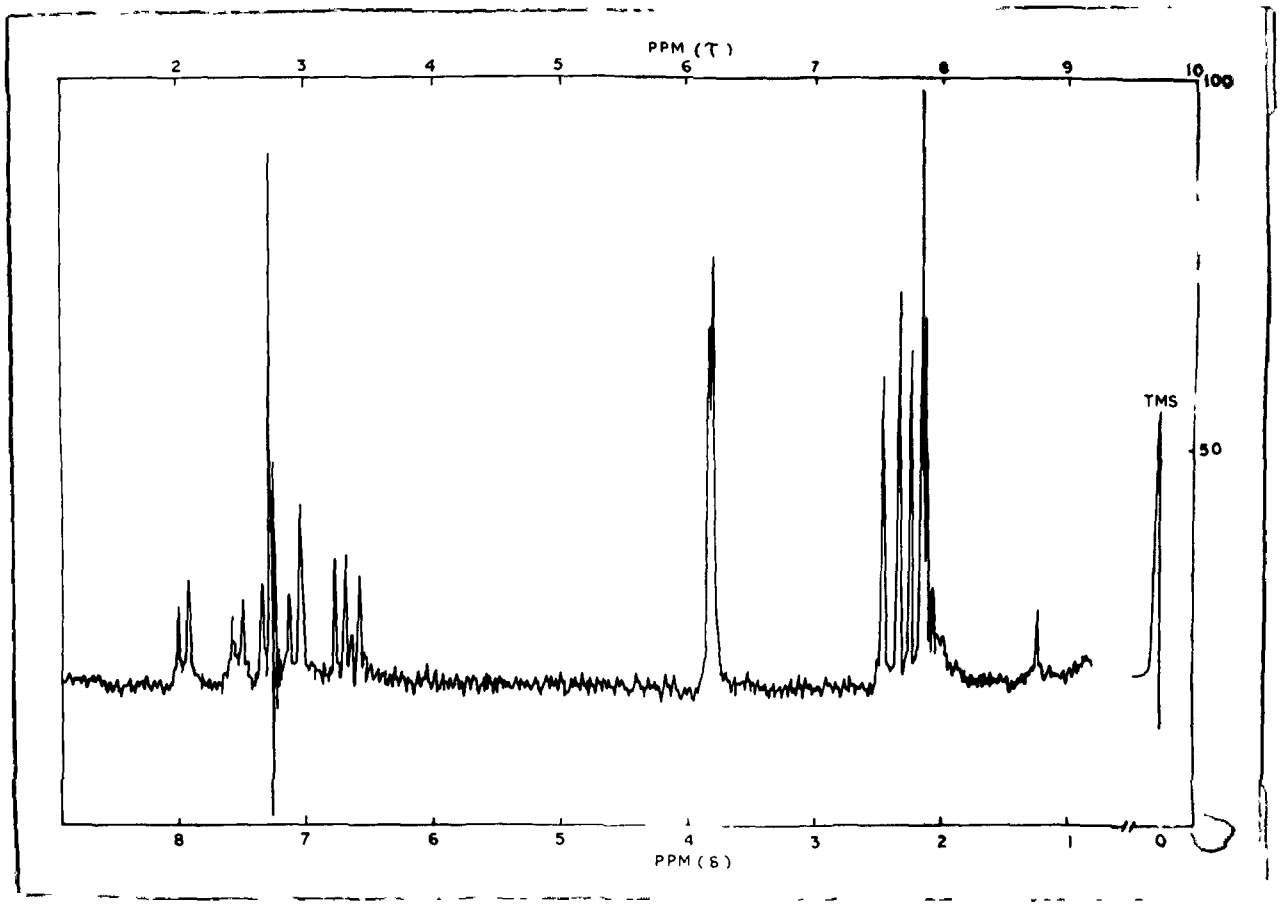


Fig. XV

4',4"',5,5"-Tetrahydroxy-7,7"-di-O-methyl-6,8"-biflavonyl (WN_3):

	m.p.		R_f	Mol.wt.
WN_3(parent)	310°		0.43	566 (M^+)
WN_3A (acetate)	169-70°	$[\alpha]_D^{34}$ -12.5($CHCl_3$)	-	734 (M^+)
$AgMe_6$(methyl ether)	160-61°		0.45	622 (M^+)

R_f value, fluorescence in UV light, mass and NMR spectra of the methyl ether ($AgMe_6$) obtained from WN_3 was identical with that of authentic agathisflavone hexamethyl ether in all respects.

The mass spectrum of WN_3 acetate (WN_3A) (m/e 734,M^+) showed it to be dimethoxytetra-acetylagathisflavone.

Although the methyl ether of WN_3 was identical in all respects with the authentic agathisflavone hexamethyl ether, the parent compound and its acetate (WN_3A) were not comparable with 4"',7-di-O-methylagathisflavone[38] (IId) and its acetate[38] (WA VIII). The NMR spectra of WN_3 and WN_3A are shown in Fig.XIV and Fig.XV respectively. The results of NMR studies of WN_3 and WN_3A are given in Table-XII.

TABLE-XII

CHEMICAL SHIFTS OF PROTONS

Assigned Positions	WN_3	WN_3A	WA VIII
H-2',6'	1.99 (2H,d) J=9 c/s	2.03	2.08
H-3',5'	2.41 (2H,d) J=9 c/s	2.70	2.73
H-2"',6"'	2.96 (2H,d) J=9 c/s	2.46	2.60
H-3"',5"'	3.18 (2H,d) J=9 c/s	2.91	3.19
H-8	3.06 (H,s)	2.96	3.01 or
H-6"	3.27 (H,s)	3.23	3.02
H-3(3")	3.38 (H,s) or 3.47 (H,s)	3.31 or 3.42	3.38 or 3.46
4'	0.7,-1.0	7.88	7.86
4"'		7.76	[6.24]
5	-3.07	7.67	7.67
5"	-3.30	7.53	7.52
7	[6.13(6H,s)]	[6.19]	[6.21]
7"		[6.17]	7.91

s= singlet, d= doublet.

Spectrum run in $CDCl_3$ at 100 Mc; TMS as internal standard = τ 10.00. Figures in parentheses show the chemical shifts of methoxyl protons.

The two sets of A_2B_2 type doublets of WN_3-acetate were comparable to those of pentaacetoxy-7-O-methylagathisflavone (WN_1-acetate) discussed earlier suggesting that no methoxy group was present at either of the 4' and 4"' positions. This was also supported by the following observations. B_2 of both A_2B_2 pairs (rings B & E) in WN_3-acetate, like WN-1 acetate moved downfield as compared to the methyl ether as well as to the parent compound. This is in contrast to tetraacetoxy-7,4"'-di-O-methylagathisflavone (WA VIII, Table-XIII)[38] where protons of

TABLE-XIII

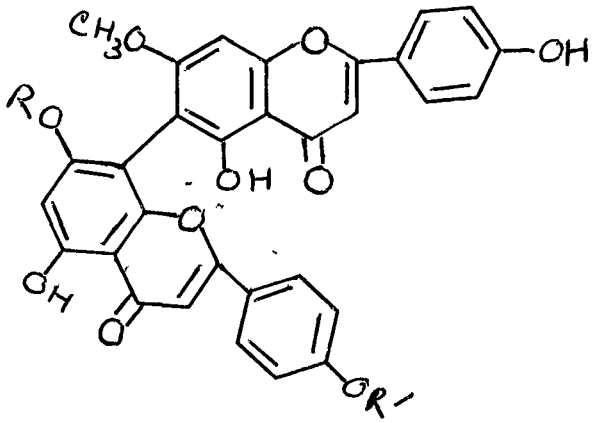
Compound		H-3',5'(B ring)	H-3"',5"' (E ring)
Pentahydroxy - 7-O-methyl- agathisflavone	Parent (WN_1)	2.95	3.38
	Methyl ether	2.99	3.22
	Acetate (WN_1A)	2.70	2.89
WN_3	Parent (WN_3)	2.41	3.18
	Methyl ether ($AgMe_6$)	2.99	3.22
	Acetate(WN_3A)	2.70	2.91
Tetrahydroxy- 4"',7-di-O-methyl- agathisflavone	Parent (WAIV)	2.91	3.07
	Methyl ether(WAIII)	2.99	3.22
	Acetate(WAVIII)	2.73	3.19

Values on τ scale

only B ring showed downfield shift but those of E ring did not move or moved very little. In the parent compound (WN_3,Table-XII)

there are two hydrogen bonded hydroxy groups at τ-3.07 and τ-3.30 for C-5 and C-5" hydroxy groups. The methoxy groups were not located at C-4' and C-4"' as shown earlier. This left the only possibility of the assignment of two methoxy groups at C-7 and C-7". This is supported by a comparison of the NMR spectra of WN_3A and WA VIII (Table-XII). Chemical shifts of H-6" at τ 3.23 in WN_3A and at τ 3.03 in WA VIII are in good accord to the presence of 7"-OMe in the former and 7"-OAc in the latter.

WN_3 is, therefore, a new optically active biflavonyl which has been assigned the structure 4',4"',5,5"-tetra-hydroxy-7,7"-di-O-methyl-6,8"-biflavonyl (IIc).



(II)

(c) R = CH_3; R' = H

(d) R = H; R' = CH_3

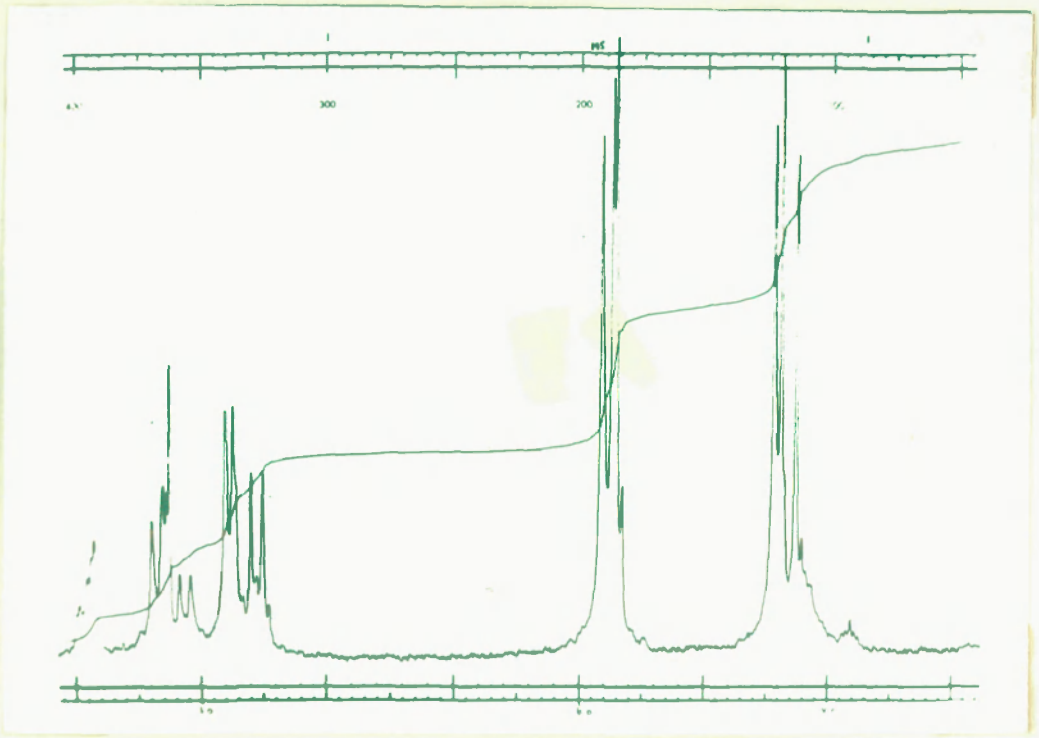
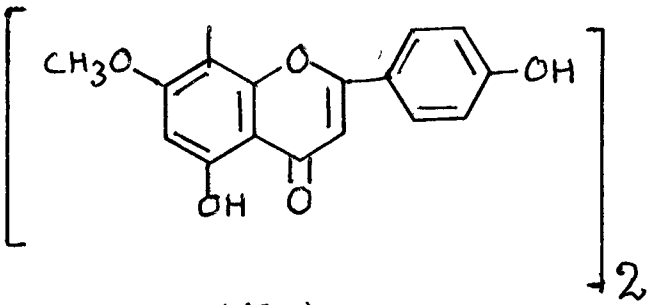


Fig. XVI

WN_4 :

WN_4 on acetylation and repeated crystallisation gave colourless needles, m.p.275-280° which was labelled as WN_4.A. The mass spectrum of WN_4.A (m/e 734,M^+) showed it to be tetraacetyl dimethyl-cupressuflavone.

The NMR spectrum of WN_4.A (Fig.XVI and Table-XV) showed two 4H doublets at τ2.95 (J=9 c/s) and τ2.66 (J=9 c/s) which attributed to H-3',5',3"',5"' and H-2',6',2"',6"' respectively. This was suggestive of a symmetrical biflavonyl. By comparison of NMR data of WN_4A_1 and WN_4M_2 with those of bisgenkwanin acetate and cupressuflavone hexamethyl ether (Table-XV), the corresponding parent compound was assigned the structure of 4',4"',5,5"-tetrahydroxy-7,7"-di-O-methylcupressuflavone (Ic)



(Ic)

WN_4 was methylated and the methyl ethers WN_4M_1 and WN_4M_2, were separated by preparative thin layer chromatography. The proton chemical shifts of WN_4M_1 (Table-XIV) and WN_4M_2(Table-XV) were identical with those of authentic amentoflavone hexamethyl ether and cupressuflavone hexamethyl ether respectively.

TABLE-XIV

CHEMICAL SHIFTS OF PROTONS OF WN_4M_1

Signals	Number of protons	J c/s	Assignment
3.24 (d)	2	9	H-3"',5"'
2.60 (d)	2	9	H-2"',6"'
2.10 (q)	1	J_1=9 J_2=3	H-6'
2.88 (d)	1	9	H-5'
2.16 (d)	1	3	H-2'
3.42, 3.48 (s)	1	-	H-3,3"
3.52 (d)	1	3	H-8
3.66 (d)	1	3	H-6
3.38 (s)	1	-	H-6"
5.94, 6.08 (s)	6	-	OMe-5,5"
6.12, 6.28 (s)	6	-	OMe-7,7"
6.25, 6.27 (s)	6	-	OMe-4',4"'

s = singlet, d = doublet, q = quartet.

Spectrum run in $CDCl_3$ at 60 Mc; TMS as an internal standard= τ10.00

TABLE-XV

CHEMICAL SHIFTS OF PROTONS

Assigned position	WE_4A_1	Cupressuflavone hexaacetate	WN_4M_2
H-2',6' (2"',6"')	2.66 (d,4H) J=9 c/s	2.71	2.70
H-3',5' (3"',5"')	2.95 (d,4H) J=9 c/s	2.99	3.23
H-6(6")	3.20 (2H,s)	2.94	3.43 or
H-3(3")	3.45 (2H,s)	3.44	3.41
4'(4"')	7.74 (6H,s)	7.75	[6.23(6H,s)]
5(5")	7.49 (6H,s)	7.52	[5.88(6H,s)]
7(7")	[6.13 (6H,s)]	7.92	[6.14(6H,s)]

s = singlet, d = doublet.

Spectrum run in $CDCl_3$ at 60 Mc; TMS as an internal standard = τ10.00

Figures in parentheses show the chemical shift of methyl protons.

The parent compounds from WN_4, however, could not be obtained in pure state either by TLC or CCD separation.

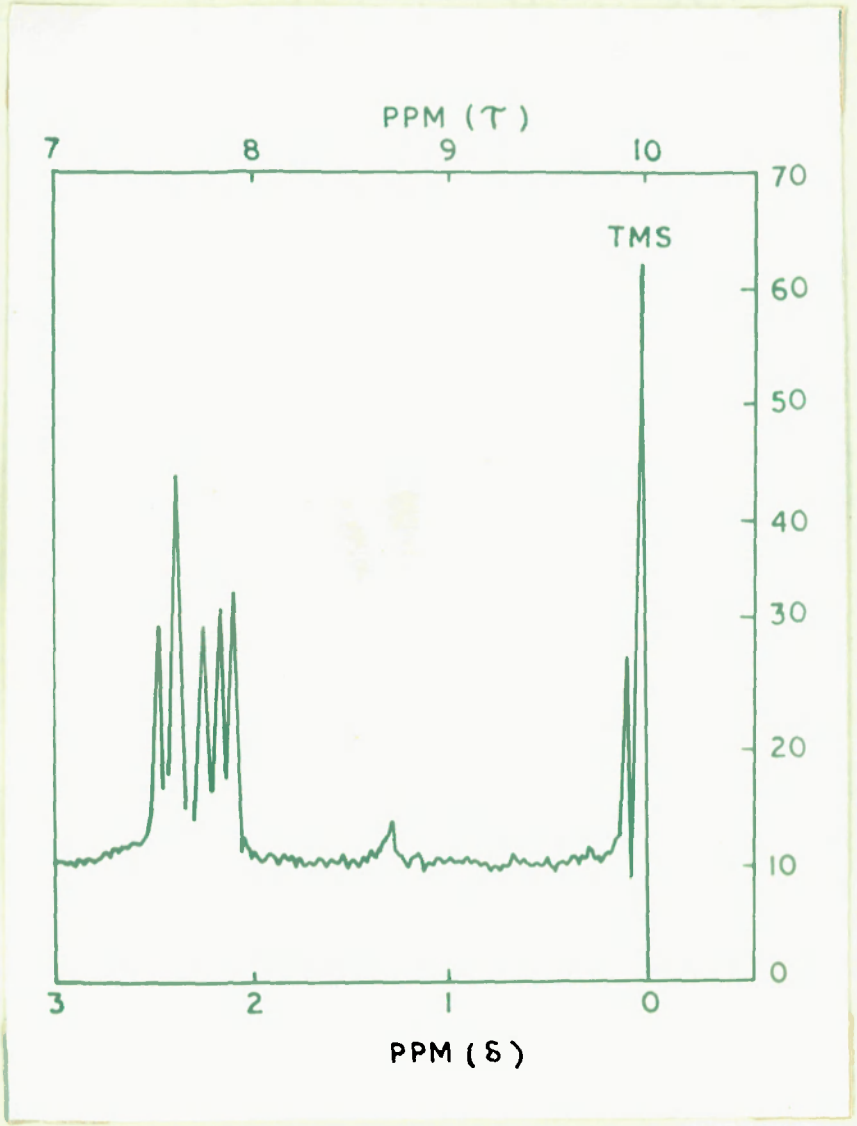


Fig. XVII

BIFLAVONYLS FROM THE LEAVES OF AGATHIS ALBA, FOXWORTHY (ARAUCARIACEAE):

The phenolic extractives of dried leaves of Agathis alba, Foxworthy (procured from National Botanical Garden, Sipore, West Bengal, India) after similar treatment as described for Araucaria bidwillii yielded six components. The components were labelled as Aa_0, Aa_1, Aa_2, Aa_3, Aa_4 and Aa_5 in the increasing order of R_f value (BPF). The usual colour reactions and ultraviolet spectra indicated all of them to be biflavonyls. Aa_5 was a minor constituent which on methylation followed by TLC examination showed the presence of amentoflavone and cupressuflavone hexamethyl ethers (R_f values and characteristic fluorescence in U.V.light).[87] R_f value considerations, however, indicated Aa_5 as a mixture of trimethyl ethers of amentoflavone and cupressuflavone.

The component Aa_0 (40 mg), was subjected to CCD separation between ethyl methyl ketone and borate buffer (pH 9.8). Aa_0X (23 mg) was obtained as major fraction. It was acetylated for NMR studies.

The results of NMR studies of Aa_0X-acetate (Aa_0XA) are given in Table-XVI and the NMR spectrum is shown in Fig. XVII.

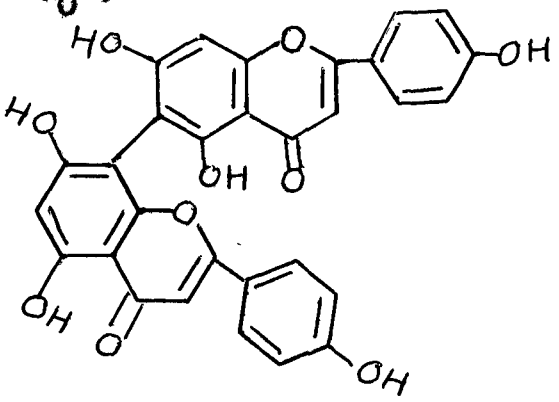
TABLE-XVI

CHEMICAL SHIFTS OF PROTONS

Compound	δ Assigned position in biflavonyl nucleus					
	4'	4"'	5	5"	7	7"
Aa_0XA	7.83	7.75	7.61	7.52	7.61	7.91
Pentaacetyl-7-O-methylagathisflavone (WA_{II})[38]	7.86	7.76	7.68	7.56	[6.20]	7.91

Spectrum run in $CDCl_3$ at 60 Mc; TMS as internal standard=τ10.00; figure in parentheses shows methoxy shift.

The comparison of NMR data of Aa_0X-acetate with that of an authentic sample of pentaacetyl-7-O-methylagathisflavone (WA_{II})[38] concluded the structure 4',4"',5,5",7,7"-hexahydroxy-6,8"-biflavonyl (IIa) for Aa_0X.



(IIa)

The structure (IIa) was further supported by the preparation of methyl ether whose NMR spectrum was comparable in all respects with that of known agathisflavone hexamethyl ether (WA_{III}).[38]

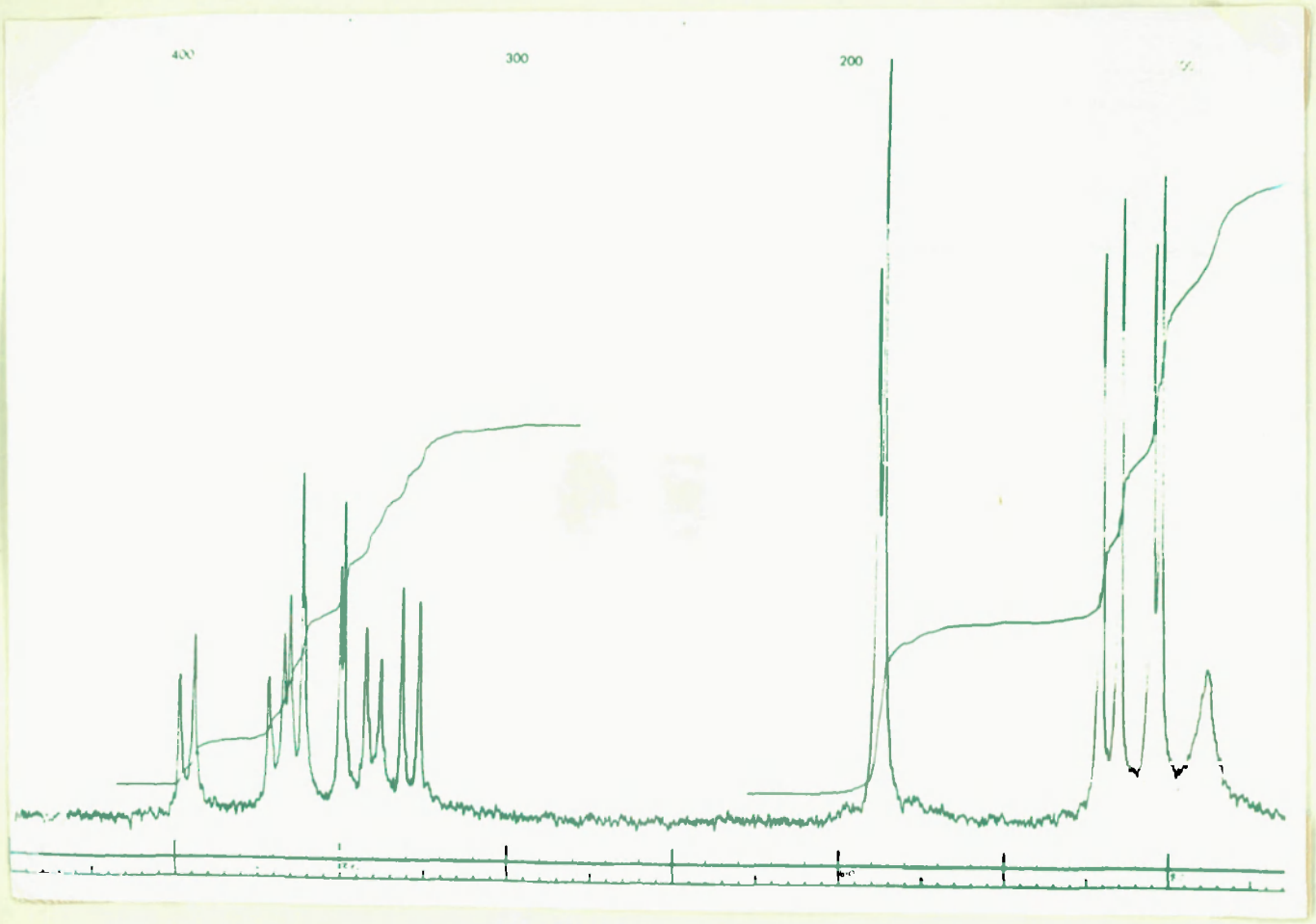


Fig. XVIII

The compound Aa_0X (IIa), thus, constitutes the first report of the isolation and characterization of the parent member of agathisflavone series. The other two minor constituents, probably amentoflavone and cupressuflavone could not be obtained in pure form.

The components Aa_1 and Aa_3 proved to be homogeneous and gave the same hexamethyl ether (AaM) which was identical in all respects with the authentic agathisflavone hexamethyl ether. The results of NMR studies of Aa_1-acetate and Aa_3-acetate were comparable with the authentic samples of 7-O-methylagathisflavone pentaacetate[38] (Table-XVII and Fig. XI) and 4"',7-di-O-methyl-agathisflavone tetraacetate[38] (Table-XVIII, Fig.XVIII) respectively.

TABLE-XVII

CHEMICAL SHIFTS OF PROTONS

Assigned position	Aa_1-Parent*	Aa_1-Acetate	AaM
H-2',6'	2.04 (2H,d) J=9 c/s	2.08	2.12
H-3',5'	2.95 (2H,d) J=9 c/s	2.62	2.99
H-2"',6"'	2.41 (2H,d) J=9 c/s	2.50	2.63
H-3"',5"'	2.88 (2H,d) J=9 c/s	2.94	3.22
H-8	3.16 (H,s)	3.00 or 3.01	3.09
H-6"	3.28 (H,s)		3.36
H-3(3")	3.40,3.61 (2H,s)	3.38,3.42	3.47,3.49
4'	0.8-1.2,	7.86(3H,s)	[6.22 or 6.24]
4"'	-3.07,-3.3 (5H)	7.76(3H,s)	
5		7.66(3H,s)	[6.41]
5"		7.56(3H,s)	[5.95]
7	[6.13 (3H,s)]	[6.20(3H,s)]	[6.12 or 6.14]
7"		7.91(3H,s)	

s= singlet, d= doublet; Spectrum run in $CDCl_3$ at 100 Mc; TMS as internal standard= τ10.00;

*Spectrum run in $(CD_3)_2CO$; figures in parentheses represent methoxy shifts.

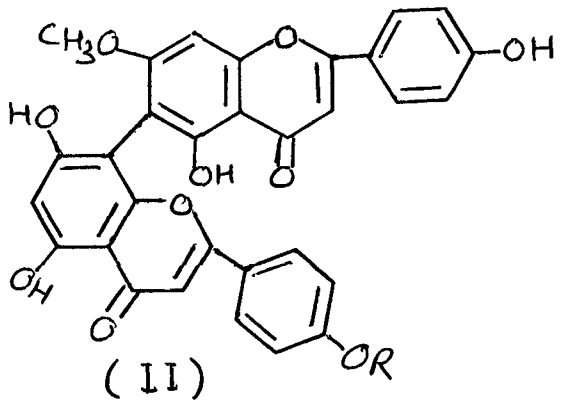
TABLE-XVIII

CHEMICAL SHIFTS OF PROTONS

Assigned position	Aa_3*-(Parent)	Aa_3-acetate (Aa_3A)
H-2',6'	2.19 (2H,d) J=9 c/s	2.08 (d)
H-3',5'	2.91 (2H,d) J=9 c/s	2.73 (d)
H-2"',6"'	2.34 (2H,d) J=9 c/s	2.60 (d)
H-3"',5"'	3.07 (2H,d) J=9 c/s	3.19 (d)
H-8	3.02 (H,s)	3.01 (s) or
H-6"	3.24 (H,s)	3.02 (s)
H-3(3")	3.33,3.59 (2H,s)	3.38,3.46 (s each)
4'	0.7-1.0 (H,s)	7.86 (s)
4"'	[6.20 (3H,s)]	[6.24 (s)]
5	-3.04 (H,s)	7.67 (s)
5"	-3.3 (H,s)	7.56 (s)
7	[6.12 (3H,s)]	[6.21 (s)]
7"	0.7-1.0 (H,s)	7.91 (s)

s= singlet, d= doublet; *- Spectrum run in $(CD_3)_2CO$; Spectrum run in $CDCl_3$ at 100 Mc; TMS as an internal standard=τ10.00; figures in parentheses show methoxy shifts.

Aa_1 and Aa_3 were, therefore, characterized as 4',4"',5,5", 7"-pentahydroxy-7-O-methyl-6,8"-biflavonyl (IIb) and 4',5,5", 7"-tetrahydroxy-4"',7-di-O-methyl-6,8"-biflavonyl (IId).



(II)

(b) R = H

(d) R = CH_3

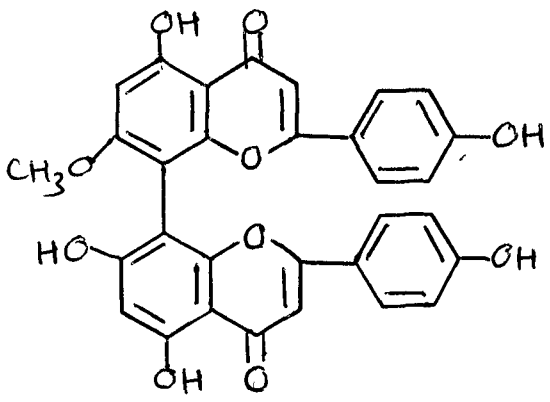
Chromatographically homogeneous fraction Aa_2 on complete methylation followed by TLC examination was found to be the mixture of hinokiflavone and monomethyl ethers of amentoflavone and cupressuflavone. CCD separation of Aa_2 between ethyl methyl ketone and borate buffer (pH 9.8) yielded $Aa_{2'}$ which was acetylated to Aa_2,A.

4',4"',5,5",7"-Pentahydroxy-7-O-methyl-8,8"-biflavonyl ($Aa_{2'}$) :

	m.p.	R_f	Mol.wt.
$Aa_{2'}$(Parent)	186-90°	0.37	552 (M^+)
Aa_2,A (acetate)	147-50°	-	762 (M^+)

The mass spectrum of Aa_2.A gave the molecular ion at m/e 762 corresponding to pentaacetyl monomethylcupressuflavone.

The results of NMR studies of Aa_2.A are given in table-XIX and the NMR spectrum is shown in Fig.XIII. The comparison of the data with that of cupressuflavone hexaacetate concludes the structure 4',4"',5,5",7"-pentahydroxy-7-O-methylcupressuflavone (Ib) for Aa_2.. This constitutes the first report of isolation and characterization of 7-O-methylcupressuflavone in nature.



(Ib)

TABLE-XIX

CHEMICAL SHIFTS OF PROTONS

Assigned positions	Aa_2, A	Cupressuflavone hexaacetate	Aa_4, A
H-3(3")	3.49 (H,s) 3.44 (H,s)	3.44 (2H,s)	3.45 (2H,s)
H-6(6")	3.21 (H,s) 2.91 (H,s)	2.94 (2H,s)	3.20 (2H,s)
H-3',5' (3"',5"')	2.97 (4H,d) J=9 c/s	2.99 (4H,d) J=9 c/s	2.95 (4H,d) J=9 c/s
H-2',6' (2"',6"')	2.67 (4H,d) J=9c/s	2.71 (4H,d) J=9 c/s	2.66 (4H,d) J=9 c/s
4'(4"')	7.73 (6H,s)	7.75 (6H,s)	7.74 (6H,s)
7(7")	[6.15 (3H,s)] 7.94 (3H,s)	7.92 (6H,s)	[6.13 (6H,s)]
5(5")	7.50 (6H,s)	7.52 (6H,s)	7.49 (6H,s)

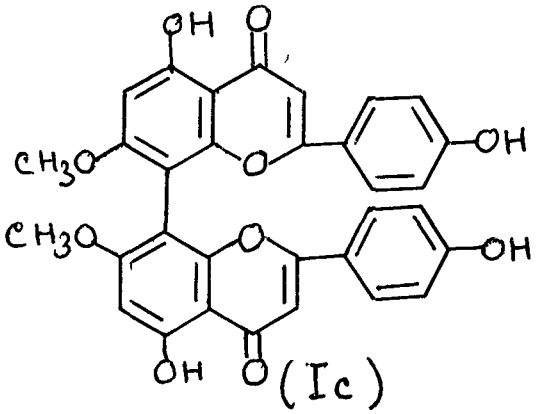
Figures in parentheses show the chemical shift of methyl protons. Spectra run in $CDCl_3$ at 60 Mc; TMS as an internal standard- τ10.00.

Aa_4, the chromatographically homogeneous fraction, on methylation and TLC examination was found to be the mixture of dimethyl ethers of amentoflavone and cupressuflavone. The CCD separation of Aa_4 between ethyl methyl ketone and borate buffer yielded Aa_4, which, after acetylation, gave Aa_4,A.

4',4"',5,5"-Tetrahydroxy-7,7"-di-O-methyl-8,8"-biflavonyl (Aa_4,) :

	m.p.	R_f	Mol.wt.
Aa_4,(parent)	300°	0.54	566 (M^+)
Aa_4/A(acetate)	258-60°	-	734 (M^+)

The mass spectrum of Aa_4,-acetate (m/e 734,M^+) indicated it to be di-O-methylcupressuflavonetetraacetate. The methylation of Aa_4, with deuterized diazomethane[118] afforded a tetradeutero-methyl ether, the mass spectrum of which showed a peak at m/e 135, due to a side phenyl fragment, $CH{\equiv}C{-}C_6H_4{-}OCD_3$ (found; 135.077, calc. for $C_9H_5D_3O$; 135.076). This observation together with the NMR results of Aa_4,A (Table-XIX) concluded structure 4',4"',5,5"-Tetrahydroxy-7,7"-di-O-methyl-8,8"-biflavonyl (Ic) for Aa_4,.



(Ic)

The other fraction, probably dimethyl ether of amentoflavone could not be obtained as a pure component.

BIFLAVONYLS FROM THE LEAVES OF ARAUCARIA COOKII R.Br.ex D.DON :

The leaves of Araucaria cookii (Araucariaceae) were procured from Sir Syed Hall Gardens, Aligarh Muslim University, Aligarh, India. Extraction of the fresh leaves followed by solvent fractionation and preparative TLC gave six fractions labelled as AC_1-AC_6. The usual colour reactions, ultraviolet spectra in ethanol and with various added diagnostic reagents indicated all of them to be flavonoids. The complexities of different fractions were studied by thin layer chromatographic examination of their fully methylated products as discussed earlier (page 91, Table-VI). The individual pure components were characterized by NMR and mass spectral studies.

The isolation and characterisation of 7,7"-di-O-methylcupressuflavone (Ic), kayaflavone (IIIl), 4',4"',7,7"-tetra-O-methyl-amentoflavone (IIIm) and 4',4"',7,7"-tetra-O-methylcupressuflavone (Ie) as major constituents of leaf extracts of Araucaria cookii has already been reported.[57] The present discussion deals with the isolation of hinokiflavone (VIIa), 7"-O-methylamentoflavone (IIId), 4"',7-di-O-methyl-agathisflavone (IId), 4',7"-di-O-methylamentoflavone (IIIi), sciadopitysin (IIIj) and 4',7,7"-tri-O-methylcupressuflavone (Id) as minor constituents.

4"',5,5",7,7"-Pentahydroxy-4'-O-6"-biflavonyl (AC_{1a}) :

	m.p.	R_f	Mol.wt.
AC_{1a} (parent)	345-46°	0.32	538 (M^+)
$AC_{1a}A$ (acetate)	240-41°	-	748 (M^+)
$AC_{1a}M$ (methyl ether)	268-70°	0.52	608 (M^+).

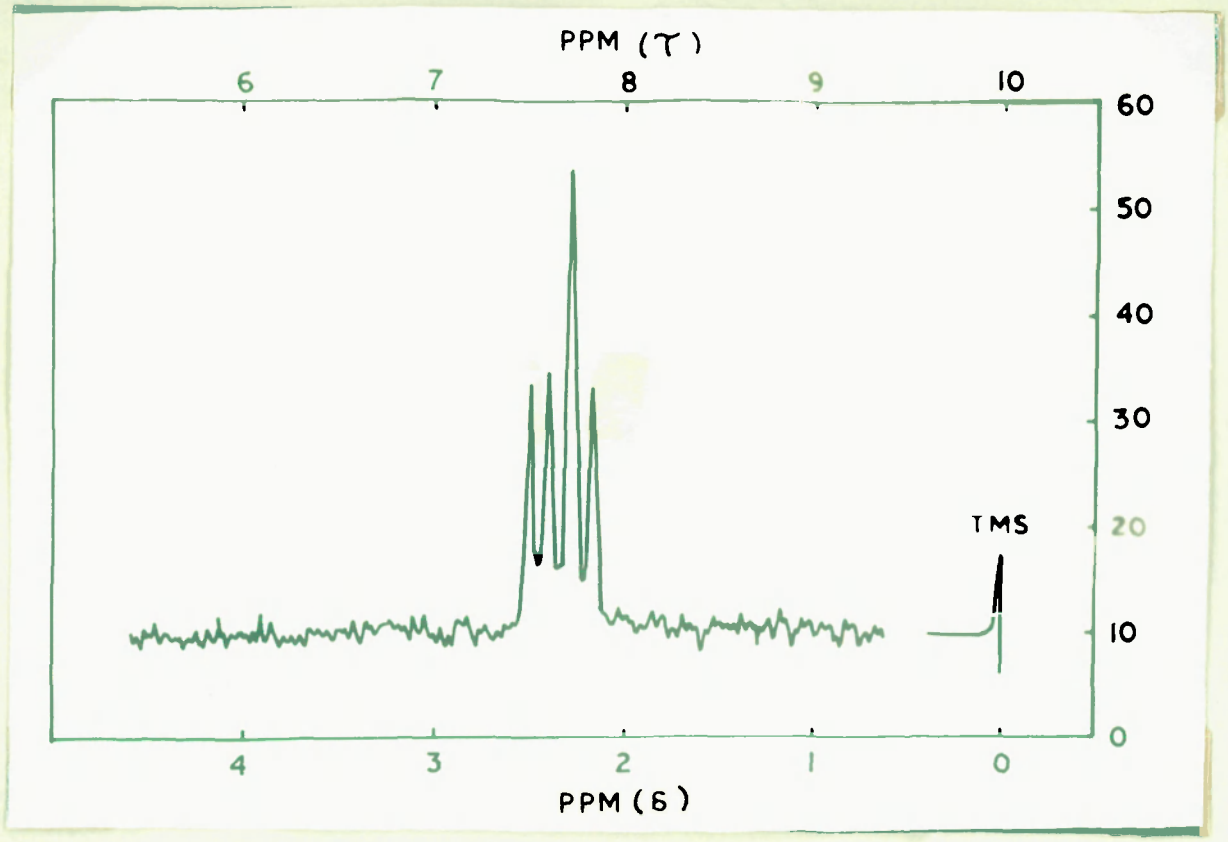


Fig. XIX

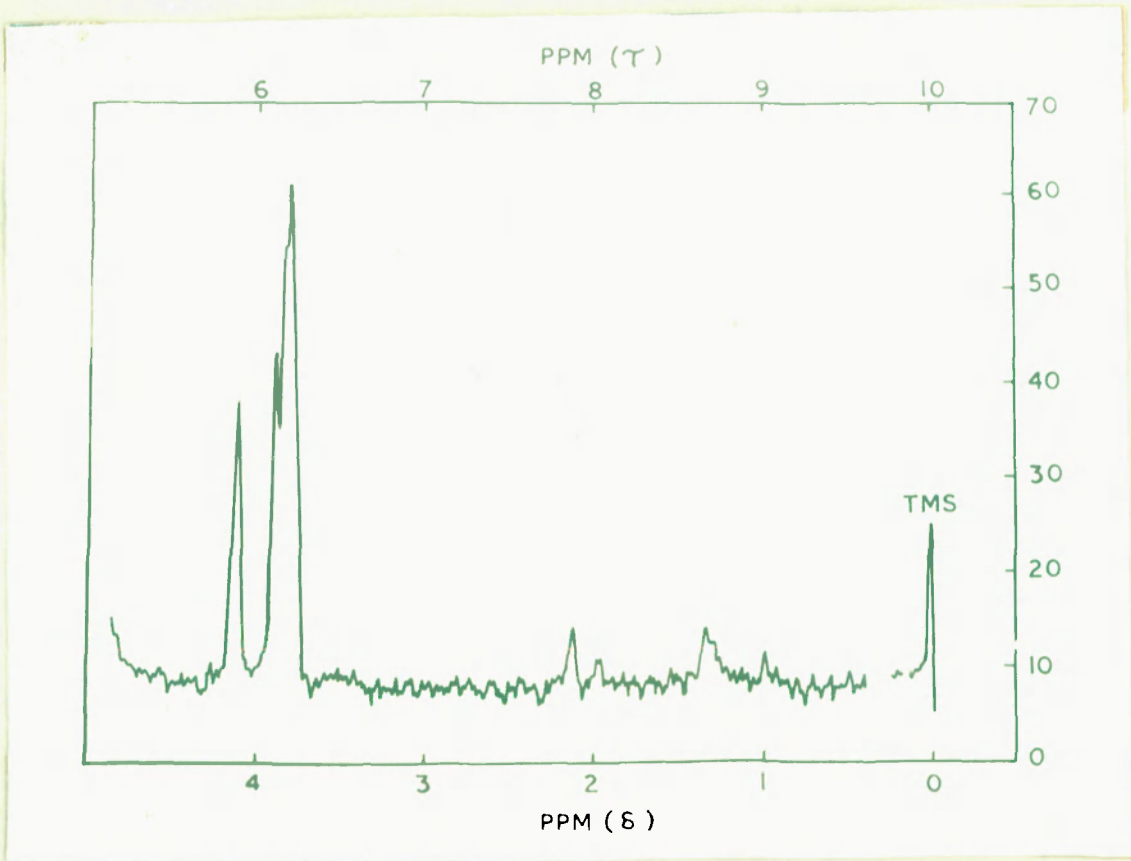
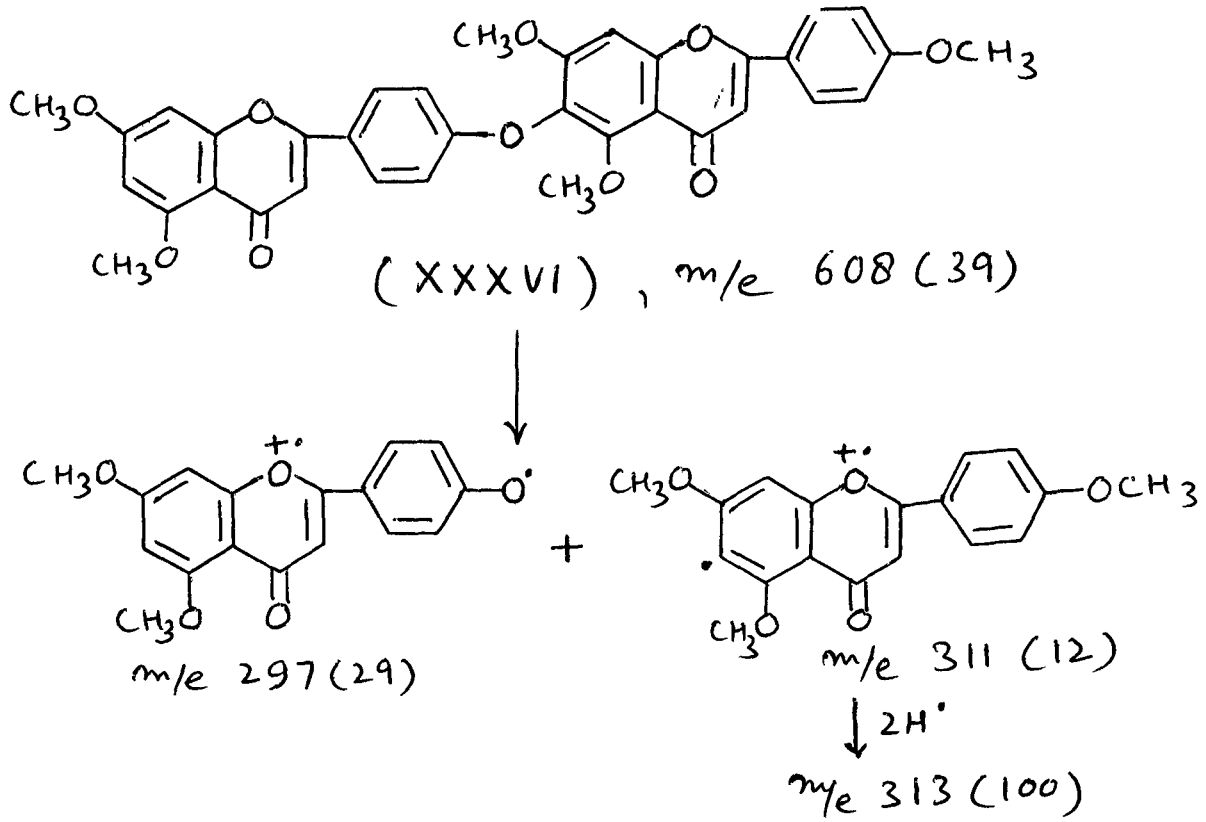


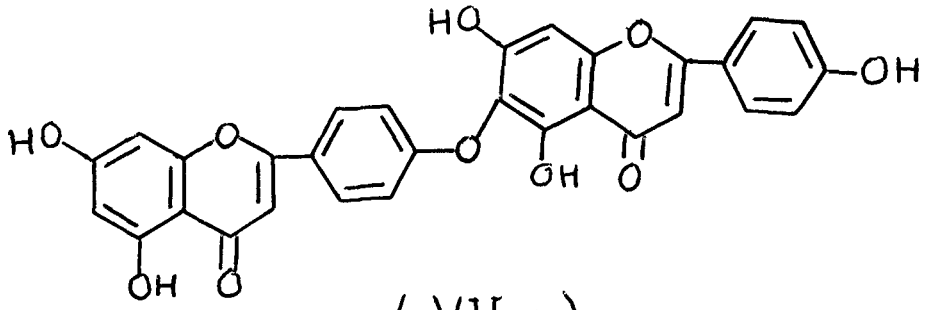
Fig. XX

The TLC examination of AC_{1a} and its methyl ether and mass spectrum of AC_{1a}-methyl ether ($AC_{1a}M$) (m/e 608, M^+) indicated that AC_{1a} may be hinokiflavone (538,M^+). The base peak at m/e 313 and a fragment at m/e 297 are in conformity with the biphenyl ether type biflavonyl structure.



The NMR spectra of $AC_{1a}A$ and $AC_{1a}M$ are shown in Fig.XIX and Fig.XX respectively.

By a comparison of NMR data (table-XX) of AC_{1a}-acetate ($AC_{1a}A$) and AC_{1a}-methyl ether ($AC_{1a}M$) with the chemical shifts of authentic compounds,[65] AC_{1a} was assigned the structure of 4"', 5,5",7,7"-penta-hydroxy-4'-O-6"-biflavonyl (VIIa).



(VII a)

TABLE-XX

NMR SIGNALS (τ SCALE) OF METHYL AND ACETYL PROTONS IN PYRIDINE SOLUTION

Compound	Assigned position in Biflavonyl Nucleus				
	4"'	5	5"	7	7"
Hinokiflavone penta-acetate(4'-O-6")	7.76	7.65	7.55	7.76	7.88
$AC_{1a}A$	7.71	7.60	7.50	7.71	7.89
Hinokiflavone penta-methyl ether (4'-O-6")	(6.23)	(6.19)	(5.92)	(6.21)	(6.14)
$AC_{1a}M$	(6.19)	(6.12)	(5.88)	(6.19)	(6.16)

Figures in parentheses show chemical shifts of methoxy protons.

4',4"',5,5",7-Pentahydroxy-7"-O-methyl-3',8"-biflavonyl (AC_{1b}) :

The CCD separation of alcohol soluble part of AC_1 yielded a pure fration, AC_{1b}. The TLC examination of AC_{1b} and its methyl ether showed that AC_{1b} may be amentoflavone monomethyl ether. The NMR spectrum of AC_{1b}-acetate ($AC_{1b}A$) is shown in

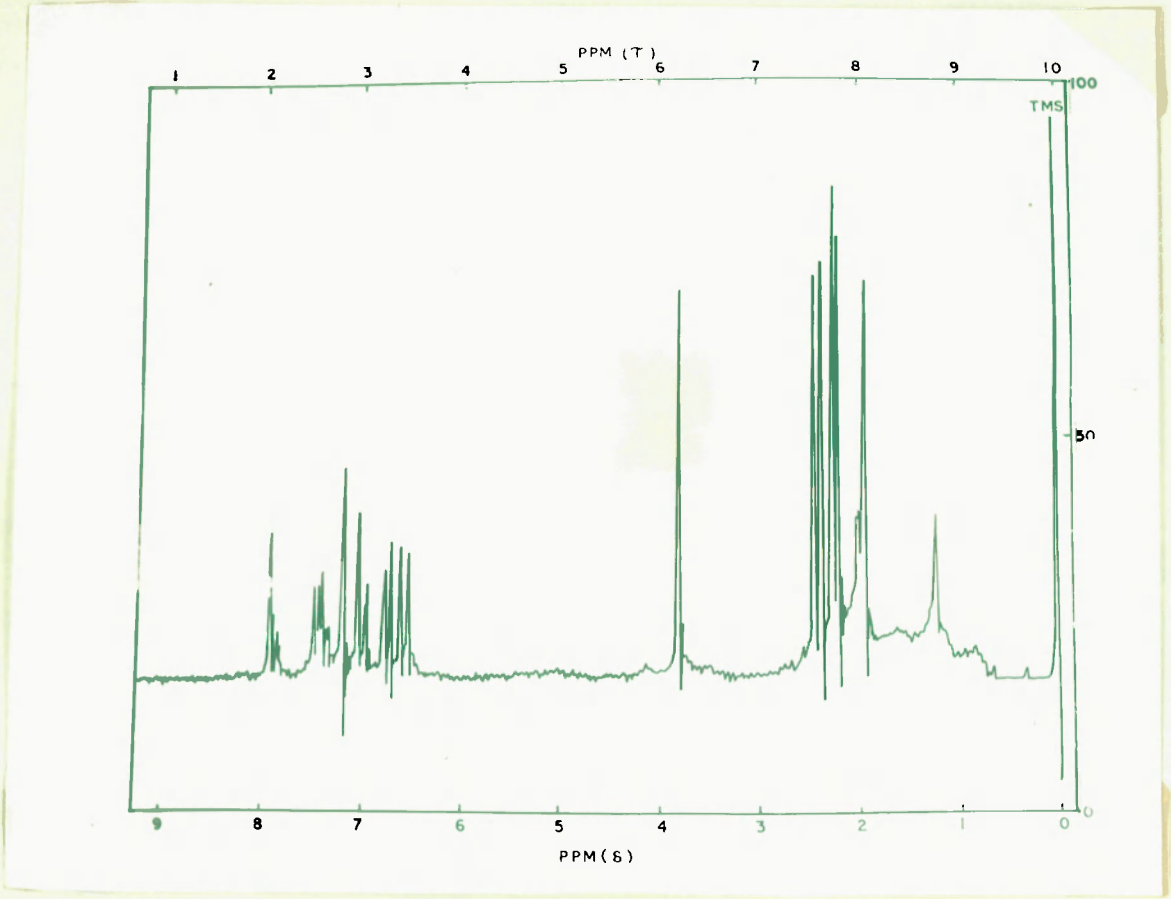
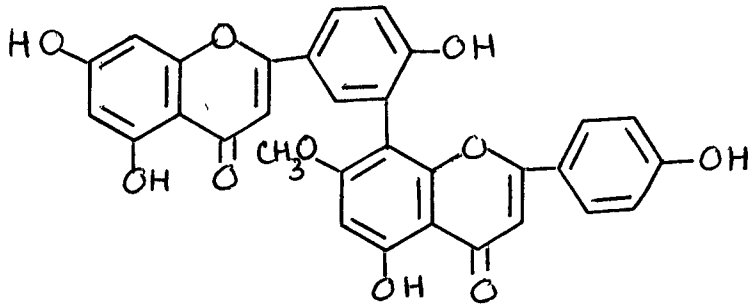


Fig. XXI

Fig.XXI. The data on comparison with amentoflavone hexamethyl ether and hexaacetate (Table-XXI) concluded the structure 4',4"', 5,5",7-pentahydroxy-7"-O-methyl-3',8"-biflavonyl (IIId) for AC_{1b}. This was previously reported as sotetsuflavone[41] which is now revised to 7"-O-methylamentoflavone (IIId), a new compound.



(IIId)

TABLE-XXI

CHEMICAL SHIFTS OF PROTONS

Assigned positions	$AC_{1b}A$	Amentoflavone hexaacetate	Amentoflavone hexamethyl ether
8	2.74, J=3 c/s (1H,d)	2.73	3.52
6	3.16, J=3 c/s (1H,d)	3.13	3.66
6"	3.23, (H,s)	2.97	3.38
6'	1.99, J_1=8 c/s (1H,q) J_2=3 c/s	1.99	2.10
5'	2.53, J=8 c/s (1H,d)	2.48	2.88
2'	1.99 J=3 c/s	1.94	2.16
2"'(6"')	2.48, J=9 c/s (2H,d)	2.50	2.62
3"'(5"')	2.95, J=9 c/s (2H,d)	2.92	3.24
3(3")	3.33, 3.41	3.30,3.32(2H,s)	3.42, 3.48
4'(4"')	7.62, 3.68	7.67,7.72	6.25, 6.27 (6H,s)
7(7")	7.95, 6.09	7.89,7.93	6.12,6.18 (6H,s)
5(5")	7.44, 7.50	7.50,7.59	5.94,6.08 (6H,s)

Spectra run in $CDCl_3$, 100 Mc, TMS as internal standard=τ10.00.

4',5,5",7"-Tetrahydroxy-4"',7-di-O-methyl-6,8"-biflavonyl (AC_2):

	M.p.	R_f	Mol.wt.
AC_2 (parent)	212-13°C	0·43	566 (M^+)
AC_2A (acetate)	181-85°C	-	734 (M^+)
AC_2M (methyl ether)	162-64°C	0·45	622 (M^+)

The TLC Examination of AC_2 and its methyl ether showed that AC_2 may be agathisflavone dimethyl ether. The mass spectra of AC_2-acetate (m/e 734,M^+) and AC_2-methyl ether (m/e 622,M^+) indicated them to be tetraacetyldimethylagathisflavone and agathisflagone hexamethyl ether respectively. The NMR data of AC_2A (Table-XXII) were identical with the authentic tetraacetyl-4"',7-di-O-methylagathisflavone.[38]

TABLE-XXII

CHEMICAL SHIFTS OF PROTONS

Assigned positions	AC_2A	AC_2M
H-2',6'	2.08 (d, J=9 c/s)	2.12
H-3',5'	2.73 (d, J=9 c/s)	2.99
H-2"',6"'	2.60 (d,J=9 c/s)	2.63
H-3"',5"'	3.19 (d,J=9 c/s)	3.22
H-8	3.02 (s)	3.09
H-6"	2.99 (s)	3.36
H-3,3"	3.38,3.46 (s)	3.47, 3.49
4'	7.86 (s)	(6.26)
4"'	(6.24)(s)	(6·22)
5	7.67 (s)	(6.41)
5"	7.56 (s)	(5.95)
7	(6.21)(s)	(6.12)
7"	7.91 (s)	(6.14)

Spectra run in $CDCl_3$, 100 Mc; TMS as internal standard-τ 10.00. Figures in parentheses represent methoxy shifts.

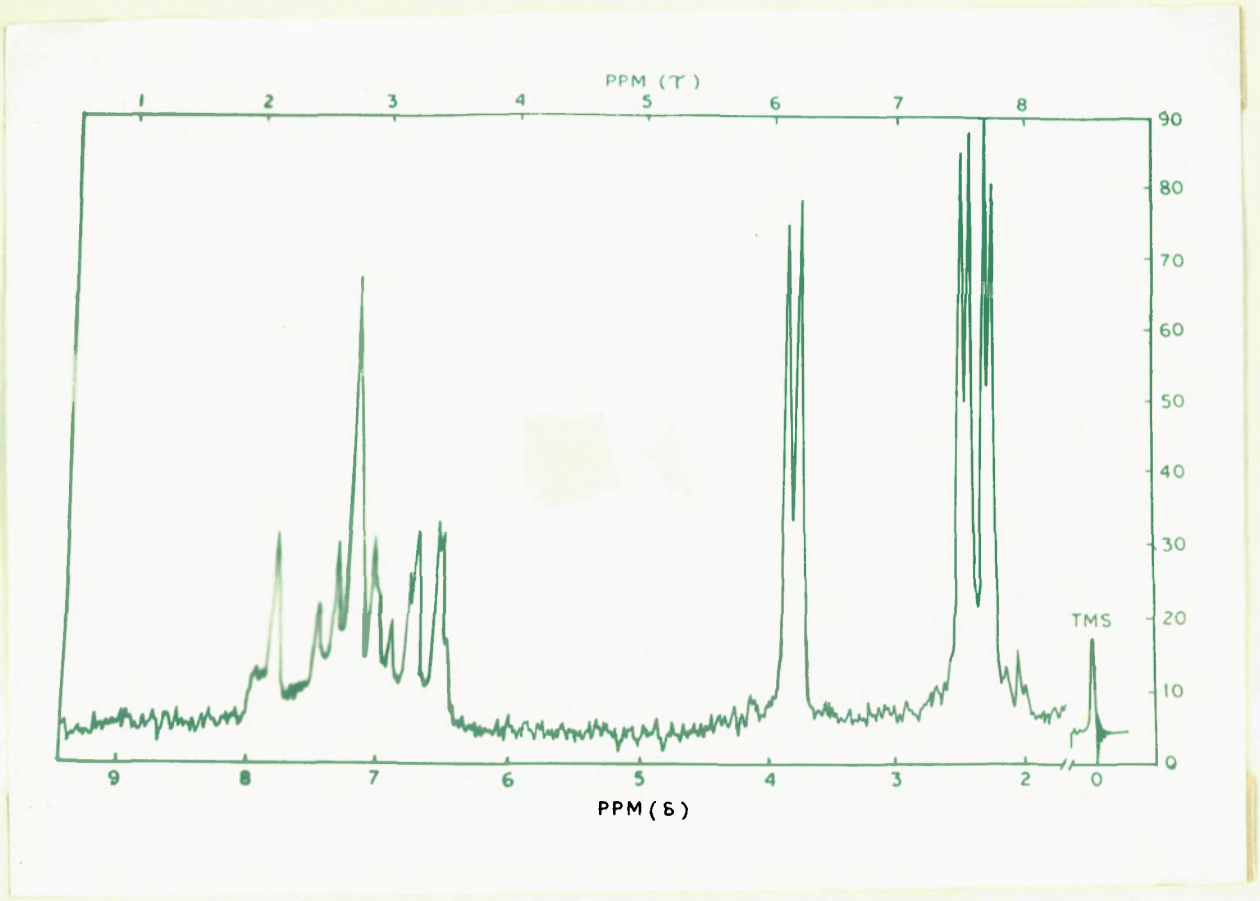
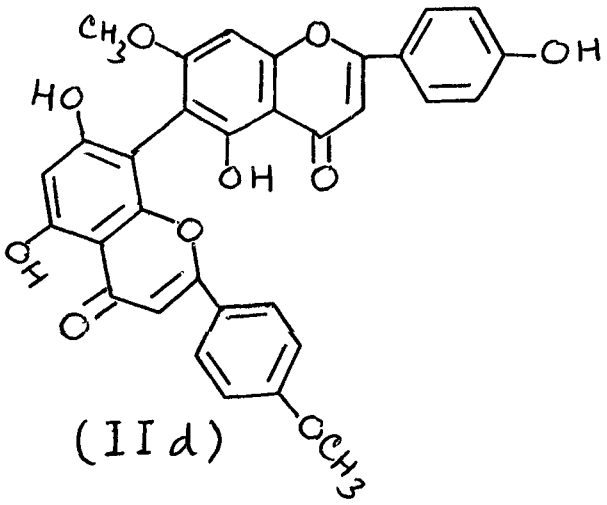


Fig. XXII

The AC_2 is, therefore, assigned the structure of 4"',7-di-O-methyl-4',5,5",7"tetrahydroxy-6,8"-biflavonyl (IId)



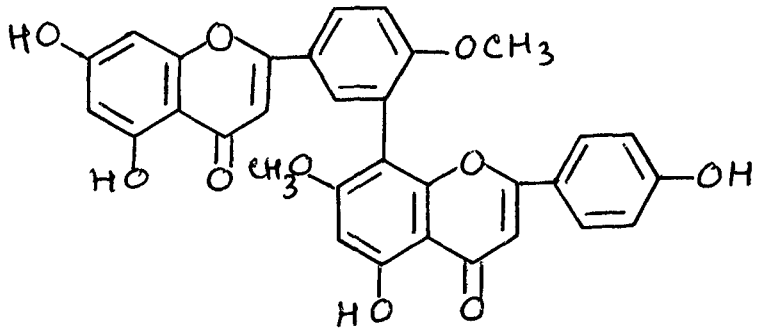
(IId)

AC_3:

AC_3 was subjected to CCD separation, whereby AC_3X_1, AC_3X_2 and AC_3X_3 were obtained as three major constituents.

4"',5,5",7-Tetrahydroxy-4',7"-di-O-methyl-3',8"-biflavonyl (AC_3X_1)

The TLC examination of AC_3X_1 and its methyl ether showed it to be dimethyl ether of amentoflavone. The NMR spectrum of AC_3X_1-acetate (AC_3X_1A) is shown in Fig.XXII. The comparison of NMR data of AC_3X_1A with the chemical shifts of acacetin acetate, ginkwanin acetate and other biflavonyls of amentoflavone series (Table-XXIII) concluded the structure 4"',5,5",7"-tetrahydroxy-4',7"-di-O-methyl-3',8"-biflavonyl (IIIi) for AC_3X_1.



(IIIi)

The remaining two fractions AC_3X_2 and AC_3X_3 could not be obtained in pure form.

TABLE-XXIII

CHEMICAL SHIFTS OF PROTONS

	8	6	6"	2',6'	5'	2"',6"'	3"',5"'	3,3"
Kayaflavone acetate	2.73 (H,d)	3.22 (H,d)	3.20 (H,s)	2.15(H,d) 2.10(H,q)	2.88 (H,d)	-	-	3.50 (H,s) 3.40 (H,s)
AC_3X_1A	2.73 (H,d)	3.20 (H,d)	3.25 (H,s)	2.08-2.1 (H,d) 2.08-2.1 (H,q)	2.87 (H,d)	2.56 (2H,d)	2.97 (2H,d)	3.47 (H,s) 3.40 (H,s)
Acacetin acetate	2.71 (H,d)	3.21 (H,d)		2.22 (2H,d)	-	-	-	3.47 (H,s)
Bisgenkwanin acetate	-	-	3.20 (H,s)	-	-	2.66 (2H,d)	2.95 (2H,d)	3.45 (2H,s)
Genkwanin acetate	-	-	3.19 (H,s)	-	-	-	-	3.49 (H,s)

— CONTINUED —

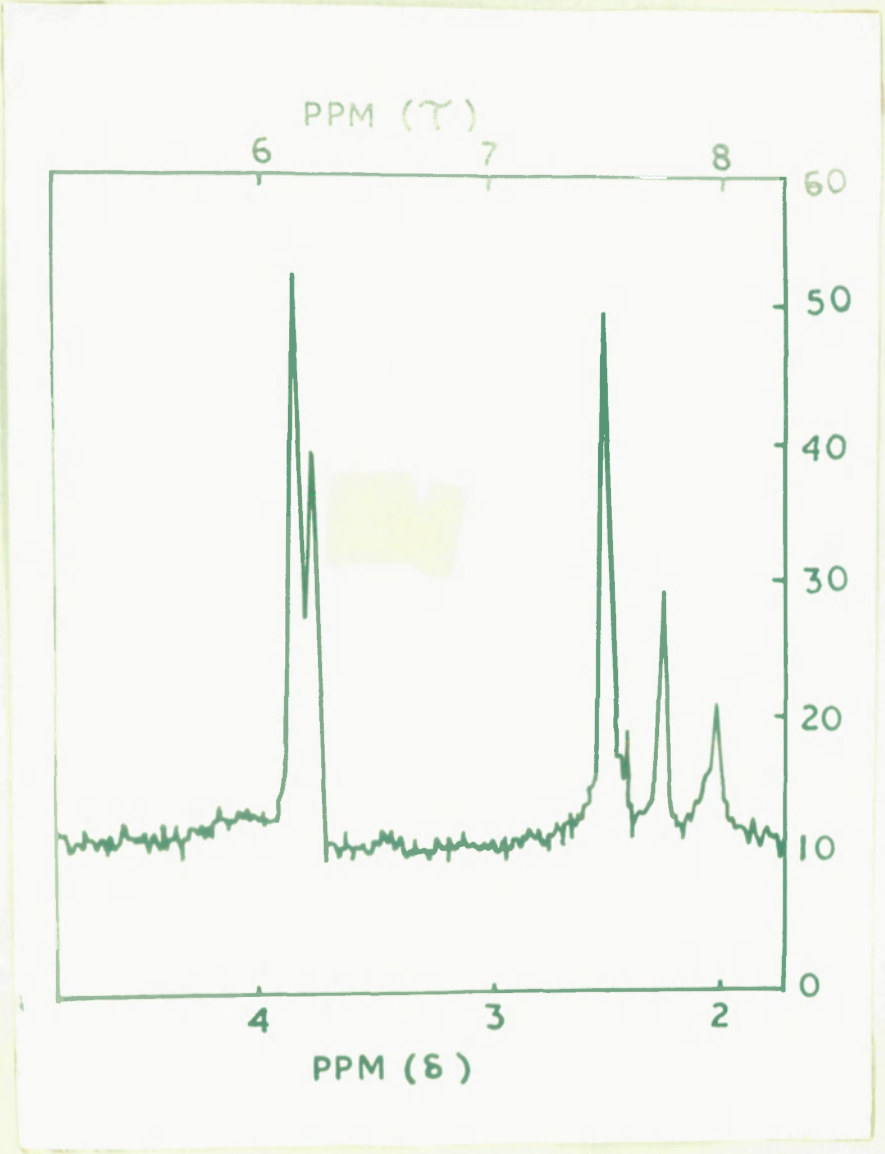


Fig. XXIII

	4',4"'	7,7"	5,5"
Ginkgetin acetate	[6.27] 7.86	[6.27] 7.90	7.48 7.48
Isoginkgetin acetate	[6.24] [6.39]	7.74 7.87	7.45 7.51
Podocarpusflavone B acetate	7.94 [6.45]	[6.27] 7.84	7.46 7.52
AC_3X_1A	[6.22] 7.73	[6.13] 7.78	7.50 7.56
Kayaflavone acetate	[6.21] [6.24]	[6.15] 7.70	7.52 7.58

All spectra run in $CDCl_3$, 100 Mc instrument (courtesy, Takeda Chemical Industries, Osaka); TMS as internal standard τ 10.00; Numbers in parentheses show chemical shifts of methoxy protons.

4"',5,5"-Trihydroxy-4',7,7"-tri-O-methyl-8,8"-biflavonyl (AC_4X_1):

The TLC examination of AC_4X_1 (R_f 0.60) and its methyl ether indicated that it may be cupressuflavone trimethyl ether. The NMR spectrum of AC_4X_1-acetate (AC_4X_1A, Table-XXIV, Fig.XXIII) was identical in all respects with the synthetic sample of triacetoxy-4',7,7"-tri-O-methylcupressuflavone.[35]

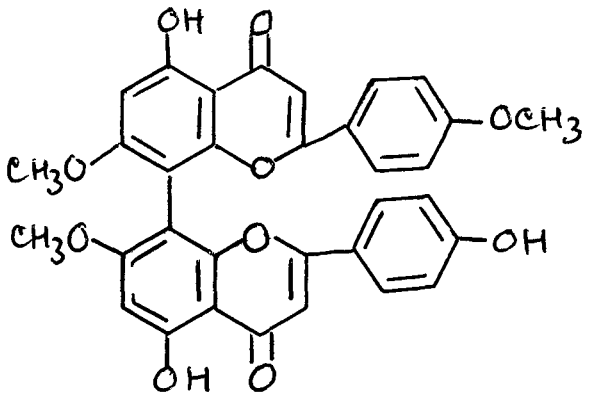
TABLE-XXIV

CHEMICAL SHIFTS OF PROTONS

Compound	Assigned position in biflavonyl nucleus		
	4',4"'	7,7"	5,5"
Cupressuflavone triacetate (synthetic)	7.73 [6.22]	[6.16]	7.48
AC_4X_1A (Natural)	7.72 [6.21]	[6.15]	7.47

Spectra run in $CDCl_3$, 60 Mc; TMS as internal standard. τ 10.0. Figures in parentheses show the chemical shifts of methoxy protons.

AC_4X_1 was, therefore, assigned the structure of 4"',5,5"-trihydroxy-4',7,7"-tri-O-methyl-8,8"-biflavonyl (Id)



(Id)

5,5",7"-Trihydroxy-4',4"',7-tri-O-methyl-3',8"-biflavonyl (AC_4X_3):

	m.p.	R_f	Mol.wt.(M^+)
AC_4X_3 (parent)	280°	0.613	580
AC_4X_3A (acetate)	252-55°		706

The TLC examination of AC_4X_3 and its methyl ether indicated it to be amentoflavonetrimethyl ether. The results of the NMR studies of AC_4X_3A (Table-XXV) were identical in all respects with the authentic sample of sciadopitysin triacetate.

TABLE-XXV

CHEMICAL SHIFTS OF PROTONS

Compound	δ Assigned position in biflavonyl nucleus					
	4'	4"'	5	5"	7	7"
Kayaflavone triacetate	[6.27]	[6.40]	7.53	7.42	7.76	[6.20]
Sciadopitysin triacetate	[6.24]	[6.42]	7.50	7.47	[6.27]	7.90
AC_4X_3A	[6.24]	[6.42]	7.52	7.50	[6.28]	7.91

Spectra run in $CDCl_3$ at 60 Mc; TMS as internal standard-τ10.00. Figures in parentheses show methoxy shifts.

The AC_4X_3 was, therefore, assigned the structure of 5,5",7"-trihydroxy-4',4"',7-tri-O-methyl-3',8"-biflavonyl (IIIj).

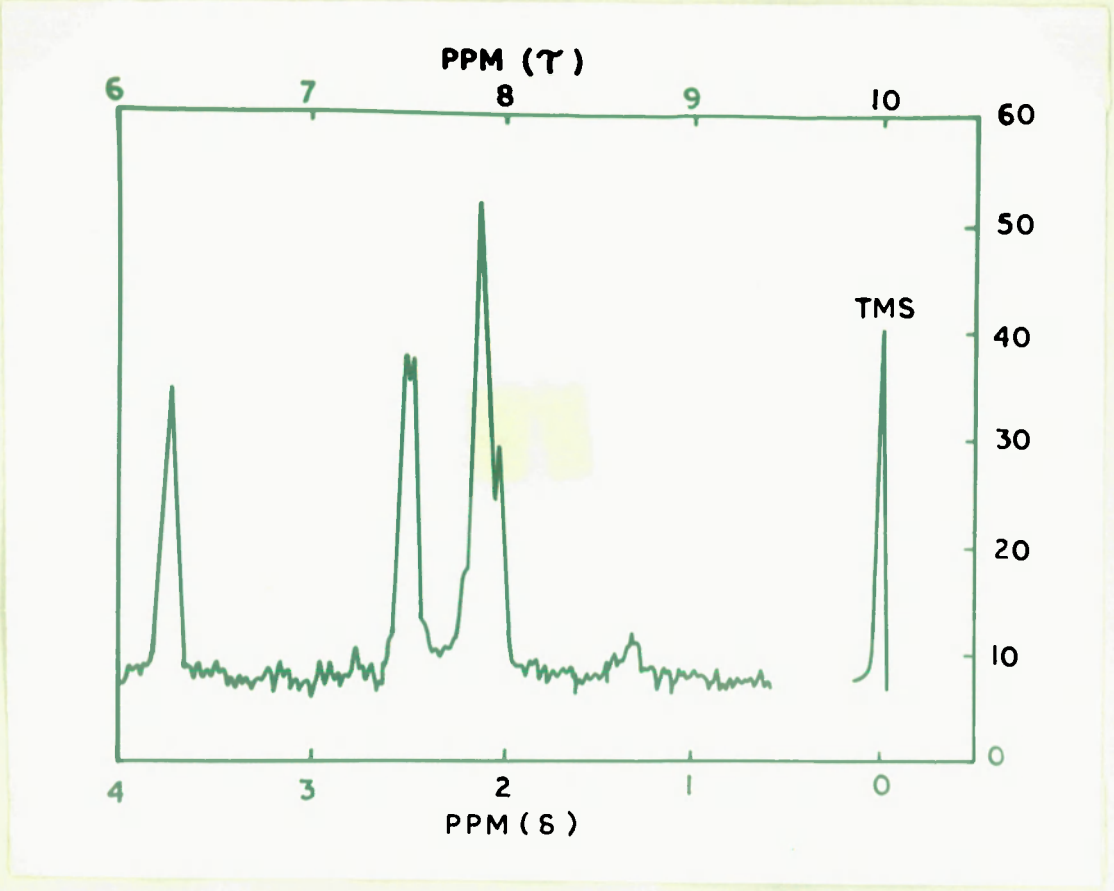
BIFLAVONYLS OF CEPHALOTAXUS DRUPACEA SIEB.& ZUCC.(CEPHALOTAXACEAE):

The conifers constitute an isolated, old and conservative group of plants dating back some two hundred million years. There are several small genera of unclear classification, and the chemical examination of these could at least give results of interest to the botanists.

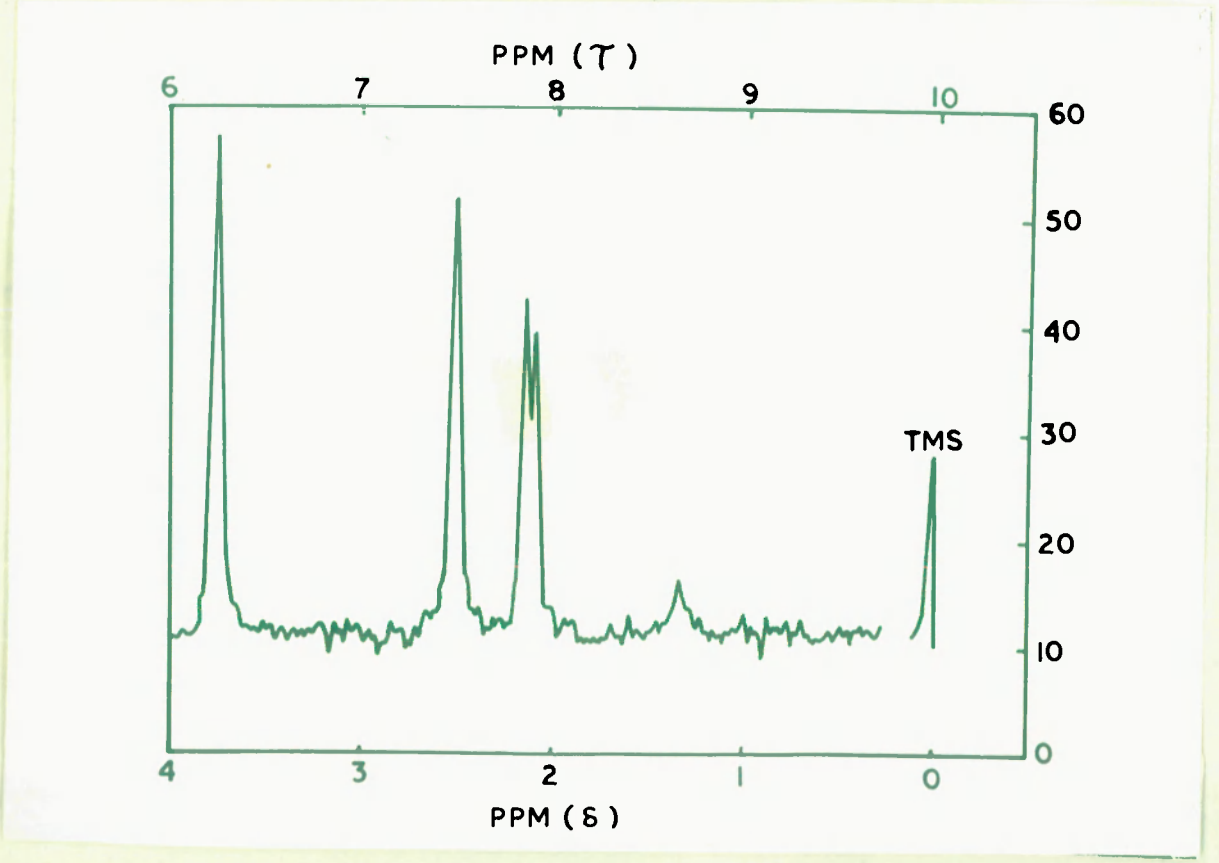
Strong fossil evidence was produced by Florin (1938-44) in support of his opinion that the Taxaceae and Cephalotaxaceae of Pilger are not true conifers but represent a collateral order Taxales.[148] The fact that biflavonyls with a few exception are restricted mainly to the leaves of gymnosperms and that specially in connection with other constituents, they are very important for their chemical classification stimulated us to carefully investigate Cephalotaxus for their biflavonyl contents.

Two Cephalotaxus species C.drupacea Sieb.& Zucc and C.nana, Nakai seem to have been investigated so far. Both of them are reported to contain Kayaflavone,[104] along with apigenin-5-rhamnoglucosyl in the former.[156]

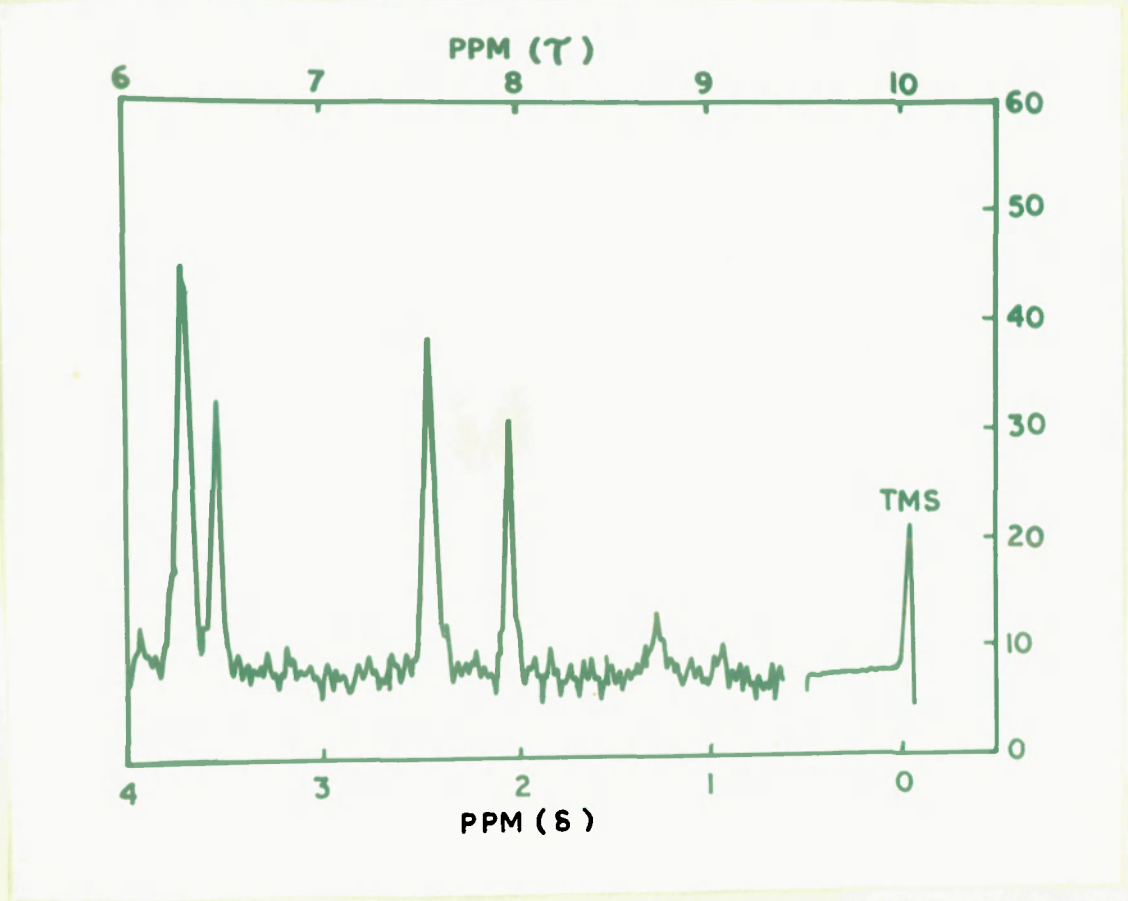
The phenolic extractives of fresh leaves by solvent fractionation and column chromatography followed by preparative thin-layer chromatography gave four components, three major and one minor. They were labelled as CT_1, CT_2, CT_3 and CT_4. The usual colour reactions and ultraviolet spectra indicated them to be flavonoids. All the components on complete methylation gave the same methyl ether (CTM) which was identified as amentoflavone hexamethyl ether by m.p., mixed m.p., R_f value (Table-XXVI), mass and NMR spectral studies (Table-XXVII).


PPM (τ)
6
7
8
9
10
60
50
40
30
20
10
0
TMS
4
3
2
1
0
PPM (δ)

XXIV


PPM (τ)
6
7
8
9
10
60
50
40
30
20
10
0
TMS
4
3
2
1
0
PPM (δ)

XXV



X XVI

The R_f value considerations of CT_1-CT_4 indicated them as amentoflavone and its mono- , di-, and tri-O-methyl derivatives respectively. CT_1 was a minor constituent which was detected as the amentoflavone (R_f value and characteristic fluorescence in UV light).

TABLE-XXVI

Compound	m.p.*	R_f value	Mol.wt. (M^+)
CT_2	300°	0.37	552
CT_2-acetate	245°	-	762
CT_3	300°	0.54	266
CT_3-acetate	256°	-	714
CT_4	295°	0.61	580
CT_4-acetate	266-67°	-	706
CTM	225°	0.40	622

* - m.p's are uncorrected.

The structures of CT_2-CT_4 were elucidated by a comparison of methoxy-, and acetoxy resonances of CT_2-CT_4 acetates with those of authentic samples (Table-XXVII). The NMR spectra of CT_2-CT_4 acetates are shown in Fig. XXIV - XXVI.

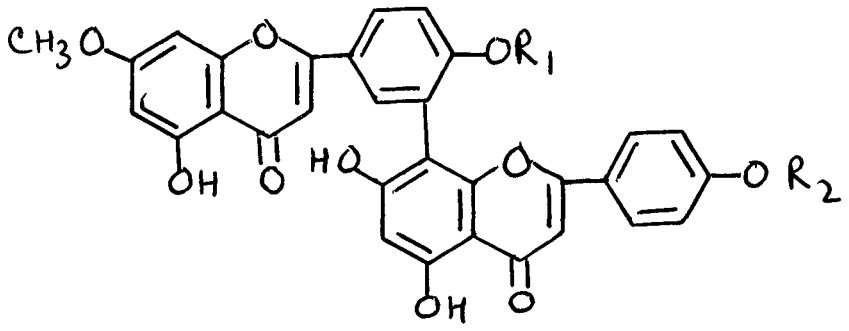
TABLE-XXVII

NMR SIGNALS (τ SCALE) OF METHYL AND ACETYL PROTONS IN PYRIDINE SOLUTION

Compound	Assigned position in biflavonyl nucleus					
	4'	4"'	5	5"	7	7"
CT_2-acetate	(7.96)	(7.87)	(7.54)	(7.49)	6.28	(7.90)
Sequoiaflavone pentaacetate	(7.95)	(7.85)	(7.53)	(7.49)	6.28	(7.90)
Bilobetin pentaacetate	6.28	(7.85)	(7.53)	(.7.49)	(7.76)	(7.90)
CT_3-acetate	6.27	(7.87)	(7.50)	(7.50)	6.27	(7.91)
Ginkgetin tetraacetate	6.27	(7.86)	(7.48)	(7.48)	6.27	(7.90)
Isoginkgetin tetraacetate	6.24	6.39	(7.51)	(7.45)	(7.74)	(7.87)
CT_4-acetate	6.24	6.43	(7.52)	(7.50)	6.28	(7.91)
Sciadopitysin triacetate	6.24	6.42	(7.50)	(7.47)	6.27	(7.90)
Kayaflavone triacetate	6.27	6.40	(7.53)	(7.42)	(7.76)	6.20

Numbers in parentheses show the chemical shifts of acetoxy protons.

The fractions CT_2-CT_4 have, thus, been assigned the structures sequoiaflavone (IIIb), ginkgetin (IIIf) and sciadopitysin (IIIj) respectively.



(III)

(b) R_1=R_2=H Sequoiaflavone

(f) R_2=H; R_1= Me Ginkgetin

(j) R_1=R_2=Me Sciadopitysin.

The presence of sciadopitysin in Cephalotaxus is noteworthy and is in contrast to the observation of Baker and Ollis,[104] that the Taxaceae are characterised by the presence of sciadopitysin whereas the Cephalotaxus yield kayaflavone. Furthermore, the non-coniferous[153] plants of Cycadales, Ginkgoales, Taxaceae and Cephalotaxaceae produce biflavonyls of only amentoflavone series. On the similar grounds, not only the separation of Sciadopityaceae from Taxodiaceae but also its inclusion with the above non-coniferous group should appear justified. The true conifers with the notable exception of Pinaceae (the inability of synthesizing biflavonyls is perhaps characteristic of the order Pinales), however, produce biflavonyls belonging to more than one series as shown in Table-XXVIII.

TABLE-XXVIII

DISTRIBUTION OF BIFLAVONYLS IN CONIFERALES

Family		Biflavonyl Type
1. Cupressaceae	...	Amentoflavone, cupressuflavone and hinokiflavone
2. Taxodiaceae	...	Amentoflavone & hinokiflavone
3. Podocarpaceae	...	Amentoflavone & hinokiflavone
4. Araucariaceae	...	Amentoflavone, Cupressuflavone, hinokiflavone & agathisflavone.

It may, therefore, be inferred that a morphological divergence in Cycadales, Ginkgoales, Taxaceae, Cephalotaxaceae and Sciadopityaceae is accompanied by 'Chemical Convergence'. It is possible that similar enzyme systems present in them are synthesizing analogous compounds.

C__O__N__C__L__U__S__I__O__N

C O N C L U S I O N

(1) BIFLAVONYLS OF THE ARAUCARIALES :

The complex mixtures of biflavonyls from the leaf extracts of Araucaria bidwillii, A.cookii, A. cunninghamii, A. alba and A.palmerstonii were examined (TLC).

(a) The presence of agathisflavone series seems to be characteristic of the order.

(b) Araucaria bidwillii, Agathis alba and Agathis palmerstonii are comparable in having biflavonyl mixtures from parent compounds to trimethyl ether while Araucaria cookii and Araucaria cunninghamii show the presence of biflavonyls from monomethyl- to tetramethyl ethers.

(2) Biflavonyls from the leaves of Araucaria bidwillii, Hooker :

Five biflavonyls have been isolated in purified form from the phenolic extractives of Araucaria bidwillii (Araucariaceae). The structures of 7,7"-di-O-methylagathisflavone, a new optically active biflavonyl and other four biflavonyls were established by ultra violet, nuclear magnetic resonance and mass spectroscopy. The interflavonyl linkage was established by studies on benzene induced solvent shifts of methoxy resonances. The following structures were assigned.

(a) 4'-O-Methylamentoflavone (Bilobetin)
(b) 7-O-Methylcupressuflavone
(c) 7-O-Methylagathisflavone
(d) 7,7"-Di-O-methylcupressuflavone (Biogenkwanin)
(e) 7,7"-Di-O-methylagathisflavone.

(3) BIFLAVONYLS FROM THE LEAVES OF AGATHIS ALBA, FOXWORTHY:

Five biflavonyls were isolated in pure form from the phenolic extractives of the leaves of Agathis alba, Foxworthy (Araucariaceae). They were characterized by UV, NMR and mass spectroscopy as:

(a) Agathisflavone
(b) 7-O-Methylagathisflavone
(c) 7-O-Methylcupressuflavone
(d) 4"',7-Di-O-methylagathisflavone
(e) 7,7"-Di-O-methylcupressuflavone.

The parent agathisflavone and 7-O-methylcupressuflavone are being reported for the first time.

(4) BIFLAVONYLS FROM THE LEAVES OF ARAUCARIA COOKII :

The phenolic extractives of Araucaria cookii yielded ten biflavonyls in pure form belonging to all the known series of biflavonyls. They were characterized by UV, NMR and mass spectroscopy. The four major constituents have already been reported.

1. 7,7"-Di-O-methylcupressuflavone
2. 4',4"',7"-Tri-O-methylamentoflavone
3. 4',4"',7,7"-Tetra-O-methylamentoflavone
4. 4',4"',7,7"-Tetra-O-methylcupressuflavone.

The following additional biflavonyl constituents have been isolated and characterized by UV, NMR and mass spectroscopy.

The minor constituent sotetsuflavone has now been revised to 7"-O-methylamentoflavone.

5. Hinokiflavone

6. 7"-O-Methylamentoflavone

7. 4"',7-Di-O-methylagathisflavone

8. 4',7"-Di-O-methylamentoflavone

9. 4',7,7"-Tri-O-methylcupressuflavone

10. Sciadopitysin.

Araucaria cookii thus constitutes a second source for the occurrence of 4',7"-di-O-methylamentoflavone and 4',7,7"-tri-O-methylcupressuflavone.

(5) BIFLAVONYLS FROM THE LEAVES OF CEPHALOTAXUS DRUPACEA SEIB. AND ZUCC.:

(a) Three biflavonyls were isolated in pure form from the phenolic extractives of Cephalotaxus drupacea, Sieb.& Zucc. They were assigned the following structures by UV, NMR and mass spectroscopy.

(a) Sequoiaflavone

(b) Ginkgetin

(c) Sciadopitysin

(b) Although it is very difficult to suggest any taxonomic correlation only on the basis of distribution of biflavonyls, but their abundance mainly in gymnosperms may help to bring about certain generalisations which can be of great help to "Chemotaxonomists."

E X P E R I M E N T A L

BIFLAVONYLS OF THE ARAUCARIALES-THIN-LAYER CHROMATOGRAPHIC EXAMINATION

All the reagents used were of 'ANALAR' grade except formic acid (E.Merck). Using a thin layer applicator (Desaga, Heidelberg) glass plates (20x20 cm) were coated with a well-stirred suspension of silica gel G (E.Merck; 50 gm-95 ml water) to give a layer approximately 0.5 mm in thickness. After drying for 2 hr at room temperature, the plates were activated at 110-120° for 1 hr and preserved in a desicator until required. Pure samples of fully methylated biflavonyls (1 mg/ml in $CHCl_3$) and parent and partial methyl ethers (1 mg/ml in pyridine) were applied with suitable micro-liter pipettes at the starting line (2 cm from the lower edge of plate and 2 cm apart from each other). The plates were mounted on stainless steel frame and placed in Desaga glass chamber 10x22x21 cm containing 200 ml. of solvent. When the solvent front travelled 15 cm from the starting line the development was interrupted and the plates were dried at room temperature. Spots were located either in UV light or by spraying $FeCl_3$-EtOH or diazotized sulphanilic acid as chromogenic reagents.

Preparation of Chromogenic reagents:

$FeCl_3$ Solution: 1% ethanolic solution of $FeCl_3$ was used as spray reagent.

Diazotized sulphanilic acid :

Cold mixture of 2N KOH (225 ml) containing 50 g sulphanilic acid and 10% $NaNO_2$ (200 ml), was added, drop-wise with stirring

to a solution of 18N H_2SO_4 (80 ml) and water (40 ml) at 0°. The precipitated p-sulphobenzene-diazonium salt was filtered off, washed successively with ice cold water, ethanol and ether and air dried. The diazotized sulphanilic acid (0.4 g) was dissolved in 100 ml of 2N NaOH and used as spray reagent.

Isolation of biflavonyls of the Araucariales:

Dried and powdered leaves (100 g) of each species after exhausting with light petrol (40-60°) in soxhlet were extracted with acetone. The concentrate after solvent fractionation followed by column chromatography on magnesium silicate (Woelm) was subjected to TLC examination. Both TLC and preparative TLC chromatograms were on silica gel G (E.Merck) using BPF (benzene-pyridine-formic acid, 36:9:5). The two dimensional chromatograms were developed (a) first in toluene-pyridine-acetic acid (10:1:1)/(20:1:1) and then in BPF and vice versa; (b) first in toluene-dimethyl formamide-acetic acid (10:1:1) and then in BPF and vice versa; and (c) both as first and second phase of the solvent system.

The methylation of pure components as well as chromatographically homogeneous mixtures was carried out using methyl iodide-potassium carbonate in dry acetone as described ~~earlier~~ in Araucaria bidwilli.

The plants investigated were as follows:-

(A) Araucaria bidwilli, Hooker
(B) Agathis palmerstonii
(C) Agathis alba, Foxworthy
(D) Araucaria cookii, R.Br.ex.D.Don
(E) Araucaria cunninghamii, Ait.

EXTRACTION OF BIFLAVONYLS FROM LEAVES OF ARAUCARIA BIDWILLI, HOOKER (ARAUCARIACEAE)

Dried and powdered leaves of Araucaria bidwillii (2 kg) were completely exhausted with petroleum ether (40-60°). The petrol extracts were concentrated first at atmospheric pressure and then under diminished pressure. An oily green residue left behind gave negative test for flavonoids and was rejected.

The petrol treated leaves were completely dried and exhausted with boiling acetone till the extract was almost colourless. The combined acetone extracts were concentrated first at atmospheric pressure and then under reduced pressure. A gummy dark green mass was obtained. This was refluxed with petroleum ether (40-60°), benzene and chloroform successively till the solvent in each case was almost colourless. The residue left behind was then treated with boiling water. The insolublo mass was dissolved in alcohol and dried under reduced pressure. A solid green residue (5 gm) thus obtained, responded to usual flavonoid colour tests.

PURIFICATION OF BIFLAVONYL MIXTURE-COLUMN CHROMATOGRAPHY:

A well stirred suspension of magnesium silicate (Woelm;200 g) in dry petroleum ether (40-60°) was poured into a column (150 cm long and 50 mm in diameter). When the adsorbent was well settled, the excess petroleum ether was allowed to pass through the column. The crude mixture of biflavonyls (5 g) was dissolved in dry acetone (80 ml) and was added to the column. After development of the column a circular filter paper was placed on the top of the

adsorbent. The column was run in with organic solvents in the increasing order of polarity. The results are given in Table-XXIX

TABLE-XXIX

Solvent	Nature of the product
1. Petroleum ether (40-60°)	greenish gummy mass
2. Benzene	green waxy product
3. Chloroform	green oily product
4. Ethyl-acetate	yellow solid (1 g)
5. Acetone	brown solid (400 mg)
6. Ethyl acetate (saturated with water)	yellow solid (800 mg)
7. Ethyl alcohol	brownish gummy mass

The fractions obtained with ethyl acetate, acetone and ethyl acetate (saturated with water) gave usual colour tests for flavones.

COLOUR TESTS :

1. Magnesium+hydrochloric acid	orange
2. Alcoholic ferric chloride	dark green
3. Zinc+hydrochloric acid	red
4. Sodium amalgam+hydrochloric acid	pinkish violet

SEPARATION OF BIFLAVONYL MIXTURE - PREPARATIVE THIN LAYER CHROMATOGRAPHY (TLC HEREAFTER):

Using thin layer spreader, (Desaga-Heidelberg) glass plates (20x20 cm) were coated with a well stirred suspension of silica gel G (E.Merck; 50 g, 95 ml water) to give a layer approximately 0.5 mm in thickness. After drying for 2 hr at room temperature, the plates were activated at 110-120° for 1 hr and preserved in a disiccator until required.

Thin layer chromatographic examination of the yellow pigments obtained as a result of elution with ethyl acetate, acetone and ethyl acetate (saturated with water) in seven solvent systems listed below indicated the presence of six compounds in each fraction. Ethyl acetate (saturated with water)fraction,however, contained lower four as major components whereas upper two were mainly present in ethyl acetate and acetone fractions. These three fractions were combined (2.29g)and subjected to separation by preparative thin-layer chromatography.

SOLVENT SYSTEMS USED :

(a)	Benzene:pyridine:formic acid (BPF)	36:9:5
(b)	Toluene:pyridine:acetic acid	5:4:1
(c)	Toluene:pyridine:acetic acid	10:1:1
(d)	Benzene:ethylacetate:acetic acid	8:5:2
(e)	Benzene:ethyl formate:pyridine:dioxan	5:2:1:2
(f)	Benzene:acetone	7:3
(g)	Benzene:acetone	5:5

In solvent system (a) the spots were compact and the differences in R_f values were so marked as to make it the developing system of choice for preparative thin layer chromatography. This solvent system was used for all the subsequent separations of biflavonyls.

Solution of biflavonyl mixture (4%) in pyridine was applied to plates with the help of mechanical applicator (Desaga,Heidelberg) 2 cm from the lower edge of the plates. The plates mounted on stainless steel frames were placed in a Desaga glass chamber (45 X 22 X 25 cm) containing 500 ml of the developing solvent

(benzene:pyridine:formic acid, 36:9:5). When the solvent front travelled 15 cm from the starting line the development was interrupted and the plates were dried at room temperature. The positions of the bands were marked in UV light. The marked pigment zones were scraped with the help of a spatula and eluted in separate columns with dry acetone. The solvent was recovered till the eluents were reduced to 20-30 ml. The addition of water yielded yellow precipitate in each case. The precipitate was filtered, washed with water and dried. Homogeneity of the pigments was checked by TLC using seven solvent systems already listed. The components were labelled as WN_0-R_f 0.16; WN_1-R_f 0.27 WN_2-R_f 0.37; WN_3-R_f 0.43; WN_4-R_f 0.54; WN_5-R_f 0.61.

WN_0 :

Being the minor constituent, WN_0 (R_f 0.16) was methylated using CH_3I and freshly ignited K_2CO_3 in dry acetone. The methylated mixture by TLC examination showed the presence of agathisflavone, amentoflavone and cupressuflavone (R_f values and characteristic fluorescence in ultra-violet light).

4',4"',5,5",7"-Pentahydroxy-7-O-methyl-6,8"-biflavonyl (WN_1) :

Crystallised as yellow needles (150 mg) from $CHCl_3$-EtOH; m.p. 310°; R_f 0.27 $\lambda_{max}^{(EtOH)}$ 278, 339 nm; (N/500 ^{NaOEt}) 285; 372, 398 nm; Mol.wt. 552 (M^+).

NMR $(CD_3)_2CO$, 100 Mc: Values on τ scale :

2.04 (d, J=9 c/s,2H,H-2',6'); 2.95 (d,J=9 c/s,2H,H-3',5'); 2.41 (d,J=9 c/s,2H,H-2"',6"'); 3.88 (d,J=9 c/s,2H,H-3"',5"'); 3.16 (S,1H,H-8); 3.28 (S,1H,H-6"); 3.40, 3.61 (S,2H-3,3"); 0.8-1.2 (2H,OH-4',4"',7"); -3.07-3.03 (2H,OH-5,5"); 6.13 (S,3H,H-7).

4',4"',5,5",7"-Pentaacetoxy-7-O-methyl-6,8"-biflavonyl (WN_1A):

A mixture of WN_1 (60 mg), pyridine (1 ml) and acetic anhydride (1 ml) was heated on a water bath for 2 hrs, cooled and poured onto crushed ice. A white solid was obtained which crystallised from $CHCl_3$-EtOH in the form of colourless needles (35 mg), m.p. 165-166° (lit. m.p. 163-168°); Mol.wt. 762 (M^+). NMR ($CDCl_3$), 100 Mc: Values on τ Scale :

2.08 (d, J=9 c/s,2H,H-2',6'); 2.62 (d,J=9 c/s, 2H,H-3',5'); 2.50 (d,J=9 c/s, 2H,H-2"',6"'); 2.94 (d,J=9 c/s, 2H,H-3"',5"'); 3.00 (s,1H,H-8); 3.01 (s,1H,H-6"); 3.38,3.42 (s,2H,H-3,3"); 6.20 (s,3H,OMe-7); 7.56 (s,3H,OAc-5"); 7.66 (s,3H,OAc-5); 7.76 (s,3H, OAc-4"'); 7.86 (s,3H,OAc-4'); 7.91 (s,3H,OAc-7").

4',4"',5,5",7,7"-Hexa-O-methyl-6,8"-biflavonyl ($WN_1M \equiv AgMe_6$):

A mixture of WN_1 (50 mg), potassium carbonate (1 g) and methyl iodide (1.5 ml) in dry acetone (150 ml) was refluxed for 22 hrs. The reaction mixture was filtered and the solvent recovered. The yellow residue left behind was taken in chloroform in a separatory funnel. The chloroform extract was washed several times with water. It was concentrated and purified by preparative thin layer chromatography. A white homogeneous product was obtained which crystallised from $CHCl_3$-MeOH as colourless needles (30 mg) m.p. 160-162° c $[\alpha]_D^{34}$ -56.5 (optically active $AgMe_6$, m.p. 162-164°; racemic form m.p. 242°), R_f 0.45 (BPF), Mol.wt. 622 (M^+).

N.M.R ($CDCl_3$), 100 M.c: Values on τ scale :

2.12 (d, J=9 c/s, 2H,H-2',6'); 2.29 (d, J=9 c/s, 2H,H-3',5'); 2.63 (d, J=9 c/s,2H,H-2"',6"'); 3.22 (d, J=9 c/s 2H,H-3"',5"'); 3.06 (s, 1H,H-8); 3.36 (s, 1H,H-6"); 3.47,3.49 (s,2H,H-3,3"); 6.26 (s,3H,OCH_3-4'); 6.22 (s,3H,OCH_3-4"'); 6.14,(s,3H,OCH_3-7); 6.12 (s,3H,OCH_3-7"); 6.41 (s, 3H,OCH_3-5); 5.95 (s,3H,OCH_3-5").

WH_2:

WH_2 (150 mg) was separated into two components WH_{2a} and WH_{2b} by CCD between ethyl methyl ketone (10 ml, equilibrated) and borate buffer (Clark-Lubs, pH 9.8, 10 ml). After 79 transfers the following two fractions were collected and acidified with hydrochloric acid. Each fraction on recovery of ethyl methyl ketone gave a pale yellow precipitate.

4"',5,5",7,7"-Pentahydroxy-4'-O-methyl-3',8"-biflavonyl (WH_{2a}, tubes 1-17):

Crystallized as yellow aggregate of needles (Ca 25 mg) from pyridine-MeOH, m.p. 300°, R_f 0.373, λ_{max}^{EtOH} 275 nm, 332 nm; Mol. wt. 552 (M^+).

4"',5,5",7,7"-Pentaacetoxy-4'-O-methyl-3',8"-biflavonyl($WH_{2a}A$):

A mixture of biflavonyl (Ca 25 mg), acetic anhydride (1 ml) and pyridine (1 ml) was heated on a water bath for 2 hrs to give an acetate which slowly crystallized from $CHCl_3$-EtOH as colourless needles (15 mg), m.p. 183-84°,Mol.wt.762(M^+). N.M.Rτppm : 6.28 (s,3H,OAc-4'); 7.07 (s,3H,OAc-4"'); 7.54(s,3H, OAc-3); 7.47 (s,3H,OAc-5"); 7.78 (s,3H,OAc-7); 7.91 (s,3H,OAc-7")

4',4"',5,5",7"-Pentahydroxy-7-O-methyl-8,8"-biflavonyl (WN_{2b}, tubes 21-25) :

Crystallized as yellow needles from $CHCl_3$- EtOH (100 mg), m.p. 186-190°, R_f 0.37; $C_{31}H_{20}O_{10}$ (Mol.wt. 552, M^+).

4',4"',5,5",7"-Pentaacetoxy-7-O-methyl-8,8"-biflavonyl ($WN_{2b}A$) :

WN_{2b} (100 mg) was acetylated with pyridine (2 ml) and acetic anhydride (2 ml). After usual work up the acetate crystallized from $CHCl_3$-EtOH as colourless needles (80 mg), m.p.147-150°, $C_{41}H_{30}O_{15}$ (Mol.wt. found; 762.159, caled.;762.158).

NMR ($CDCl_3$) 100 Mc; Values on τ scale :

2.67 (d, J=9 c/s, 4H,H-2',6',2"',6"'); 2.97 (d, J=9 c/s, 4H,H-3',5',3"',5"'); 3.21, 2.91 (s, 2H,H-6,6"); 3.49, 3.44 (s,2H, H-3,3"); 6.15 (s,3H, OMe-7); 7.50 (s,3H each, OAc-5,5"); 7.73 (s,3H each, OAc-4',4"'); 7.94 (s,3H,OAc-7").

4',4"',5,5"-Tetrahydroxy-7,7"-di-O-methyl-6,8"-biflavonyl (WN_3) :

Crystallized as yellow needles (160 mg) from $CHCl_3$-EtOH; m.p.310°; R_f 0.43 (BPF); Mol.wt. 566 (M^+).

NMR ($CDCl_3$) 100 Mc: Values on τ scale :

1.99 (d, J=9 c/s, 2H,H-2',6'); 2.41 (d, J=9 c/s, 2H,H-3', 5'); 2.96 (d, J=9c/s, 2H,H-2"',6"'); 3.18(d, J=9 c/s, 2H,H-3"',5"'); 3.06 (s, 1H,H-8); 3.27 (s,1H,H-6"); 3.38, 3.47 (s, 1H each, H-3,3"); 6.13 (s, 6H, OMe-7,7"); 0.7, -1.0 (2H, OH-4',4"'), -3.07, -3.30 (2H, OH-5,5").

4',4"',5,5"-Tetraacetoxy-7,7"-di-O-methyl-6,8"biflavonyl (WN_3A):

A mixture of WN_3 (60 mg), pyridine (1 ml) and acetic anhydride (1 ml) was heated on a water bath for 2 hrs., cooled and poured onto crushed ice. A white solid so obtained was filtered and washed with water. It crystallized from $CHCl_3$-EtOH in the form of colourless needles (40 mg), m.p.169-170°, $[\alpha]_D^{34}$ -12.50 ($CHCl_3$), Mol.wt. 734 (M^+).

NMR ($CDCl_3$), 100 Mc: Values on τ scale :

2.03 (d, J=9 c/s, 2H,H-2',6'); 2.46 (d, J=9 c/s, 2H,H-2"', 6"'); 2.70 (d, J=9 c/s, 2H,H-3',5'); 2.91 (d, J=9 c/s,2H,H-3"', 5"'); 2.90,(s,1H,H-8); 3.23 (s,1H,H-6"); 3.31,3.42 (s,2H,H-3, 3"); 6.17,(s,3H,OCH_3-7"); 6.19 (s,3H,OCH_3-7); 7.53 (s,3H,OCH_3-3"); 7.67,(s,3H,OAc-5); 7.76 (s,3H,OAc-4"'); 7.88 (s,3H,OAc-4').

4',4"',5,5",7,7"-Hexa-O-methyl-6,8"-biflavonyl (WN_3M= $AcMe_6$):

A mixture of WN_3 (50 mg), potassium carbonate (1 g) and methyl iodide (1.5 ml) in dry acetone (150 ml) was refluxed for 18 hrs. After usual work up it cryotallized from $CHCl_3$-MeOH as colourless needles (35 mg) m.p. 160-162°(optically active $AcMe_6$, m.p. 162-164°;(racemic form m.p. 242°), R_f 0.45 (BPF), Mol. wt. 622 (M^+).

NMR ($CDCl_3$), 100 Mc: Values on τ scale :

2.12 (d, J=9 c/s,2H,H-2',6'); 2.99 (d, J=9 c/s, 2H,H-3',5'); 2.63 (d, J=9 c/s, 2H,H-2"',6"'); 3.22 (d, J=9 c/s,2H,H-3"',5"'); 3.09 (s,1H,H-8); 3.36 (s,1H,H-6"); 3.47, 3.49 (s,2H,H-3,3"); 6.20 (s,3H,OCH_3-4'); 6.22 (s,3H,OCH_3-4"'); 6.14 (s,3H,OCH_3-7); 6.12 (s,3H,OCH_3-7"); 6.41 (s,3H,OCH_3-5); 5.95 (s,3H,OCH_3-5").

WN_4 (250 mg) :

WN_4 was found to be the mixture of dimethyl ethers of amentoflavone and cupressuflavone by TLC examination of its completely methylated product.

4',4"',5,5"-Tetraacetoxy-7,7"-di-O-methyl-8,8"-biflavonyl (WN_4A_1) :

WN_4 (100 mg) was acetylated using pyridine (1.5 ml) and acetic anhydride (1.5 ml). After usual work up, a dull white solid was obtained. On repeated crystallizations from $CHCl_3$-EtOH, it gave colourless needles (60 mg), m.p. 275-280°, Mol.wt.734 (M^+), $C_{32}H_{22}O_{10}$.

NMR ($CDCl_3$), 100 Mc: Values on τ scale :

2.66 (d, J=9 c/s,4H,H-2',6',2"',6"'); 2.95 (d, J= 9 c/s, 4H,H-3',5',3"',5"'); 3.20 (s, 2H,H-6,6"); 3.45 (s,2H,H-3,3"); 6.13 (s,3H each, OCH_3-7,7"); 7.49 (s,3H each, OAc-5,5"); 7.74 (s, 3H each, OAc-4,4"').

WN_4-Methyl ether :

A mixture of WN_4 (150 mg), potassium carbonate (2 mg) and methyl iodide (2.5 ml) in dry acetone (150 ml) was refluxed for 12 hrs. After usual work up and TLC examination (BPF), methylated product showed presence of hexamethyl ethers of amentoflavone and cupressuflavone (R_f value and characteristic shade in U.V.light) Ca. 50:50. Both the components were separated using preparative TLC and labelled as WN_4M_1 (60 mg) and WN_4M_2 (60 mg).

4',4"',5,5",7,7"-Hexa-O-methyl-3',8"-biflavonyl (WN_4M_1) :

Crystallised from chloroform-methanol as colourless needles (60 mg), m.p.225°, R_f 0.40, λ_{max}^{EtOH} 267 nm, 328 nm, Mol.wt.622(M^+).

NMR ($CDCl_3$), 100 Mc: Values on τ scale :

3.24 (d, J=9 c/s, 2H, H-3"',5"'); 2.60 (d, J=9 c/s, 2H, H-2"',6"'); 2.10 (q J_1=9 c/s and J_2= 3 c/s, 1H, H-6'); 2.88 (d, J=9 c/s, 1H, H-5'); 2.16 (d, J=3 c/s, 1H, H-2'); 3.42, 3.48 (s, 2H, H-3,3"); 3.52 (d, J=3 c/s, 1H, H-8); 3.66 (d, J=3 c/s, 1H, H-6); 3.38 (s, 1H, H-6"); 5.94, 6.08 (s, 3H each, OMe-5,5"); 6.12, 6.28 (s, 3H each, OMe-7,7"), 6.25, 6.27 (s, 3H each, OMe-4',4"').

4',4"',5,5",7,7"-Hexa-O-methyl-8,8"-biflavonyl (WN_4M_2) :

Crystallised from $CHCl_3$-Methanol as ~~colourless needles~~ (60 mg), m.p. 299° R_f 0.43, Mol.wt. 622 (M^+).

NMR ($CDCl_3$), 100 Mc: Values on τ scale :

2.70 (d, J=9 c/s, 4H, H-2',6',2"',6"'); 3.23 (d, J=9 c/s, 4H, H-3',5',3"',5"'); 3.43, 3.41 (s, H-6,6",3,3"); 6.23 (s, 3H each, OCH_3-4',4"'); 6.14 (s, 3H each, OCH_3-7,7"); 5.88 (s, 3H each, OCH_3-5,5").

WN_5 :

WN_5, R_f 0.61 a minor constituent on methylation with MeI-K_2CO_3 followed by TLC examination showed presence of trimethyl ethers of amentoflavone and cupressuflavone (R_f value and characteristic shade in U.V.light).

EXTRACTION OF BIFLAVONYL FROM LEAVES OF AGATHIS ALBA, FOXWORTHY:

Dried and powdered leaves of Agathis alba, Foxworthy (2 kg) were completely exhausted with petroleum ether (40-60°). The petrol treated leaves were completely dried and exhausted with boiling acetone till the extract was almost colourless. The combined acetone extracts were concentrated first at atmospheric pressure to give a dark viscous mass. This was refluxed successively with petroleum ether (40-60°), benzene and chloroform till the solvent in each case was almost colourless. The residue left behind was then treated with boiling water. The insoluble mass was dissolved in alcohol and dried under reduced pressure. A solid green residue (5 gm) thus obtained responded to usual colour tests for flavonoids.

PURIFICATION OF BIFLAVONYL MIXTURE-COLUMN CHROMATOGRAPHY :

The crude mixture of biflavonyls (4 gm) was dissolved in dry acetone (50 ml) and was added to a column (150 cm long and 50 mm in diameter) containing magnesium silicate (Woelm 140 gm) as adsorbent in petroleum ether. After development of the column, it was eluted with organic solvents in the increasing order of polarity. The results are given below in Table-XXX.

TABLE-XXX

Solvent	Nature of the product
1. Petroleum ether (40-60°)	green oily product
2. Benzene	green gummy mass
3. Chloroform	green oily product
4. Ethyl acetate	yellow solid (1 g)
5. Acetone	-do- (100mgs)
6. Ethyl acetate (saturated with water)	-do- (1 g)
7. Ethyl alcohol	brown waxy product

The fractions obtained with ethyl acetate, acetone and ethyl acetate (saturated with water) gave usual colour tests for flavonoids.

SEPARATION OF BIFLAVONYLS - TLC :

The three fractions obtained with ethyl acetate, acetone and ethyl acetate (saturated with water) were combined. The biflavonyl mixture (2 gm) was dissolved in dry pyridine (50 ml) and separated into six components by preparative thin layer chromatography (silica gel; NCL-Poona). The homogeneity of these components was again checked by TLC (BPF). The components were labelled as Aa_0-R_f 0.16; Aa_1-R_f 0.27; Aa_2-R_f 0.37; Aa_3-R_f 0.43; Aa_4 R_f 0.54 Aa_5 R_f 0.61.

Aa_0 :

Being the minor constituent, Aa_0 (R_f 0.16) was methylated using methyl iodide and freshly ignited potassium carbonate in dry acetone. The methylated mixture by TLC examination showed the presence of agathisflavone, amentoflavone and cupressuflavone (R_f values and characteristic fluorescence in U.V.light).

The CCD separation of Aa_0 (80 mg) between ethyl methyl ketone and borate buffer (pH 9.1, 200 transfers) gave following three fractions: Aa_0X (21-75, all pale yellow), Aa_0Y (76-115, centering at about No.90), and Aa_0Z (116-160, centering at about No.130).

4',4''',5,5''',7,7''-Hexaacetoxy-6,8''-biflavonyl (Aa_0XA) :

A mixture of Aa_0X (18 mg) acetic anhydride (1 ml) and

pyridine (1 ml) was heated on a water bath for 2 hrs. After usual work up it crystallized from $CHCl_3$-EtOH (15 mg), Mol.wt.790(M^+

NMR ($CDCl_3$) 60MC : Values on τ scale :

2.00-3.4 (12H,aromatic protons); 7.52 (s, 3H,OAc-5"); 7.61 (s,3H each, OAc-5,7); 7.75 (s,3H,OAc-4"'); 7.83 (s,3H,OAc-4'); 7.91 (s,3H,OAc-7").

NMR (Pyridine), 60 MC : Values on τ scale:

7.45 (s,3H,OAc-5"); 7.55 (s,3H,OAc-5); 7.65 (s,3H,OAc-7); 7.68 (s,3H,OAc-4"'); 7.72 (s,3H,OAc-4'); 7.78 (s,3H,OAc-7").

4',4"',5,5",7"-Pentahydroxy-7-O-methyl-6,8"-biflavonyl (Aa_1) :

Crystallized as yellow needles (160 mg) from $CHCl_3$-EtOH; m.p. 310°, R_f 0.27; λ max (EtOH) 278,339 nm; (N/500 NaOEt) 285, 372, 398nm; Mol.wt. 552 (M^+).

NMR, $(CD_3)_2CO$,100 Mc: Values on τ scale :

2.04 (d,J=9 c/s,2H,H-2',6'); 2.95 (d, J=9 c/s,2H,H-3',5'); 2.41 (d, J=9 c/s, 2H,H-2"',6"'); 2.88 (d, J=9 c/s, 2H,H-3"',5"'); 3.16 (s,1H,H-8); 3.28 (s,1H,H-6"); 3.40, 3.61 (s,2H,H-3,3"); 0.8-1.2 (3H,OH-4',4"',7"); -3.07, -3.5 (2H,OH-5,5"); 6.13 (s,3H, OCH_3-7).

4',4"',5,5",7"-Pentaacetoxy-7-O-methyl-6,8"-biflavonyl (Aa_1A) :

A mixture of Aa_1 (50 mg), pyridine (1 ml) and acetic anhydride (1 ml) was heated on a water bath for 2 hrs, cooled and poured onto crushed ice. A white solid so obtained filtered washed

with water and crystallised from $CHCl_3$-EtOH in the form of colourless needles (30 mg), m.p. 165-166° (lit.m.p.163-168°); Mol.wt. 762 (M^+).

NMR ($CDCl_3$), 100 Mc: Values on τ scale :

2.08 (d,J=9 c/s,2H,H-2',6'); 2.62 (d, J= 9 c/s, 2H,H-3',5'); 2.50 (d, J=9 c/s, 2H,H-2"',6"'); 2.94 (d, J=9 c/s, 2H,H-3"',5"'); 3.00 (s, 1H,H-8); 3.01 (s,1H,H-6"); 3.38, 3.42 (s,2H,H-3,3"); 6.20 (s,3H,OMe-7); 7.56 (s,3H,OAc-5"); 7.66 (s,3H,OAc-5); 7.76 (s,3H,OAc-4"'); 7.86 (s,3H,OAc-4'); 7.91 (s,3H,OAc-7").

4',4"',5,5",7,7"-Hexa-O-methyl-6,8"-biflavonyl (AaM) :

A mixture of Aa_1 (50 mg), potassium carbonate (1 g) and methyl iodide (1.5 ml) in dry acetone (150 ml) was refluxed for 20 hrs. The reaction mixture was filtered and the solvent recovered. The yellow residue after usual work up crystallised from $CHCl_3$-MeOH as colourless needles (30 mg) m.p.160-162°, R_f 0.45 (BPF); Mol.wt. 622 (M^+).

NMR ($CDCl_3$), 100 Mc: Values on τ scale :

2.12 (d, J=9 c/s, 2H,H-2',6'); 2.99 (d, J=9 c/s,2H,H-3',5'); 2.63 (d,J=9 c/s,2H,H-2"',6"'); 3.22 (d, J=9 c/s,2H,H-3"',5"'); 3.09 (s,1H,H-8); 3.36 (s,1H,H-6"); 3.47,3.49 (s,2H,H-3,3"); 6.12,6.14 (s,3H each, OCH_3-7,7"); 6.22, 6.24 (s,3H each, OCH_3-4', 4"'); 6.41 (s,3H,H-5); 5.95 (s, 3H, H-5").

Aa_2 :

Aa_2 was found to be the mixture of hinokiflavone and monomethyl ethers of amentoflavone and cupressuflavone by TLC examination of its completely methylated product.

Aa_2 (150 mg) was subjected to CCD separation between ethyl methyl ketone and borate buffer (9.8 pH). After 79 transfers and usual work up fraction Aa_2, could be obtained in pure form.

4',4"',5,5",7"-Pentahydroxy-7-O-methyl-8,8"-biflavonyl (Aa_2, tubes 20-25):

Crystallized as yellow needles from $CHCl_3$-EtOH (80 mg); m.p. 186-190°; R_f 0.37; $C_{31}H_{20}O_{10}$ (Mol.wt. 552, M^+).

4', 4"', 5, 5",7"-Pentaacetoxy-7-O-methyl-8,8"-biflavonyl (Aa2'A) :

Aa_2, (80 mg) was acetylated with pyridine (2 ml) and acetic anhydride (2 ml). After usual work up the acetate crystallized from $CHCl_3$-EtOH as colourless needles (60 mg), m.p. 147-150°, $C_{41}H_{30}O_{15}$ (Mol.wt., found, 762.159; Calcd., 762.158).

NMR ($CDCl_3$), 100 Mc: Values on τ scale :

2.67 (d, J=9 c/s, 4H,H-2',6',2"',6"'); 2.97 (d, J=9 c/s, 4H,H-3',5',3"',5"'); 3.21, 2.91 (s, 2H,H-6,6"); 3.49, 3.44 (s,2H, H-3,3"); 6.15 (s,3H,OMe-7); 7.50 (s, 3H each, OAc-5,5"); 7.73 (s,3H each, OAc-4',4"'); 7.94 (s, 3H,OAc-7").

4',5,5",7"-Tetrahydroxy-4"',7-di-O-methyl-6,8"-biflavonyl (Aa_3) :

Crystallized from pyridine-EtOH as yellow needles (160 mg), m.p. 212-213°, λ max (EtOH), 277, 337 nm; (M/500 NaOEt) 287, 382 (inflex), 403 nm, $C_{32}H_{22}O_{10}$ (Mol.wt. 566.1213448).

NMR $(CD_3)_2CO$, 100 MH$_z$: Values on scale :

2.19 (d,J=9 c/s,2H,H-2',6'); 2.91 (d,J=9 c/s,2H,H-3',5'); 2.34 (d, J= 9 c/s,2H,H-2"',6"'); 3.07 (d, J=9 c/s, 2H,H-3"',5"'); 3.02 (s,1H,H-8); 3.24 (s,1H,H-6"); 3.33, 3.59 (s,1H each,H-3, 3"); 0.7-1.0 (s,1H,OH-4'); 6.26 (s,3H,OCH_3-4"'); 6.11 (s,3H,OCH_3-7); 0.7-1.0 (s.1H,OH-7"); -3.04, -3.3 (s,1H each, OH-5,5").

4',5,5",7"-Tetraacetyl-4"'-7-O-methyl-6,8"-biflavonyl (Aa_3A):

A mixture of Aa_3 (60 mg), pyridine (1 ml) and acetic anhydride (1 ml) was heated on a water bath for 2 hrs, cooled and poured onto crushed ice. After usual work up it crystallised from $CHCl_3$-EtOH in the form of colourless needles (40 mg), m.p. 181-85° $C_{40}H_{30}O_{14}$ (Mol.wt. 734.162447).

NMR ($CDCl_3$), 100 Mc: Values on τ scale :

2.08 (d, J=9 c/s, 2H, H-2',6'); 2.73 (d, J=9 c/s, 2H, H-3',5'); 2.60 (d, J=9 c/s, 2H, H-2"',6"'); 3.19 (d, J=9 c/s, 2H, H-3"',5"'); 3.01, 3.02 (s, 1H each, H-6",8); 3.58, 3.46 (s, 1H each, H-3,3"); 6.21 (s, 3H, OCH_3-7); 6.24 (s, 3H, OCH_3-4"'); 7.86 (s, 3H, OAc-4'); 7.91 (s, 3H, OAc-7"); 7.67 (s, 3H, OAc-5); 7.56 (s, 3H, OAc-5").

4',4"',5,5",7,7"-Hexa-O-methylagathisflavone (Aa_3M-AaM) :

A mixture of Aa_3 (60 mg), freshly ignited potassium carbonate (1.5 g) and methyl iodide (1.5 ml) in dry acetone (150 ml) was refluxed for 20 hrs. After usual work up it crystallized from $CHCl_3$-MeOH as colourless needles (40 mg) m.p. 162-164° (racemic form m.p. 242°); R_f 0.45 (BFF); Mol.wt. 622 (M^+).

NMR ($CDCl_3$), 100 Mc: Values on τ scale :

2.12 (d, J=9 c/s, 2H, H-2',6'); 2.99 (d, J=9 c/s, 2H, H-3',5'); 2.63 (d, J=9 c/s, 2H, H-2"',6"'); 3.22 (d, J=9 c/s, 2H, H-3"',5"'); 3.09 (s, 1H, H-8); 3.36 (s, 1H, H-6"); 3.47, 3.49 (s, 2H, H-3,3"); 6.12, 6.14 (s, 3H each, OCH_3-7,7"); 6.22, 6.26 (s, 3H each, OCH_3-4', 4"'); 6.41 (s, 3H, H-5); 5.95 (s, 3H, H-5").

Aa_4:

Aa_4-R_f 0.54, was found to be the mixture of dimethyl ethers of amentoflavone and cupressuflavone by TLC examination of its completely methylated product.

4',4"',5,5"-Tetraacetoxy-7,7"-di-O-methyl-8,8"-biflavonyl ($Aa_4'A$):

Aa_4 (100 mg) was acetylated using pyridine (1.5 ml) and acetic anhydride (1.5 ml). After usual work up a brown solid was obtained. On repeated crystallisations from $CHCl_3$-EtOH, it gave colourless needles (65 mg), m.p. 275-280°, Mol.wt. 734 (M^+).

NMR ($CDCl_3$), 100 Mc: Values on τ scale :

2.66 (d, J=9 c/s, 4H, H-2',2"',6"'); 2.95 (d, J=9 c/s, 4H, H-3',5',3"',5"'); 3.20 (s, 2H, H-6"); 3.45 (s,2H, H-3,3"); 6.13 (s, 3H each, OCH_3-7,7"); 7.49 (s, 3H each, OAc-5,5"); 7.74 (s, 3H each, OAc-4',4"').

Aa_5:

Aa_5-R_f 0.61, a minor component on methylation with MeI-K_2CO_3 followed by TLC examination (BPF) showed presence of trimethyl ether of amentoflavone and cupressuflavone (R_f value and characteristic shade in U.V. light).

EXTRACTION OF BIFLAVONYLS FROM LEAVES OF ARAUCARIA COOKII :

The defatted leaves of Araucaria cookii (4 kg) were completely dried and exhausted with boiling acetone till the extract was almost colourless. The combined acetone extract were concentrated. The concentrate was purified as described earlier by solvent fractionation and column chromatography on magnesium silicate (Woelm). The yellow solid (6 g) gave usual colour tests for flavones.

SEPARATION OF BIFLAVONYL MIXTURE- PREPARATIVE TLC :

Thin layer chromatographic examination of yellow pigments by methods mentioned earlier indicated the presence of six compounds (BPF). The crude solid was dissolved in pyridine and subjected to preparative TLC. The individual components were separated and their homogeniety checked as usual. The components were labelled as AC_1-R_f 0.37; AC_2-R_f 0.43; AC_3-R_f 0.54; AC_4-R_f 0.61; AC_5-R_f 0.75 and AC_6-R_f 76. All the components gave usual colour tests for flavones. The complexities of different fractions were studied by TLC examination of their fully methylated products as discussed earlier (Table- VI page 9).

AC_1 :

AC_1 (180 mg) was refluxed in ethanol. Ethanol insoluble part (80 mg) was labelled as AC_{1a}. Ethanol soluble part (100 mg) was subjected to CCD (282 transfers) between ethyl methyl ketone and borate buffer (pH 9.3). The main part was recovered from tubes 141-185 (80 mg) and labelled as AC_{1b}.

4"',5,5",7,7"-Pentahydroxy-4'-O-6"-biflavonyl (AC_{1a}) :

AC_{1a} (80 mg) crystallised from pyridine-ethanol pale yellow crystals, m.p. 345-46°, R_f 0.37 , Mol.wt. 538 (M+).

4"',5,5",7,7"-Pentaacetyl-4'-O-6"-biflavonyl ($AC_{1a}A$):

A mixture of AC_{1a} (40 mg), pyridine (1 ml) and acetic anhydride (1 ml) was heated on water bath for 2 hrs. After usual work up it crystallised from methanol-chloroform (30mg) m.p. 240-41°, Mol.wt. 748 (M+).

NMR (Pyridine) 60 Mc ; Values on τ scale :

7.50 (s,3H,OAc-5"); 7.60 (s,3H, OAc-5); 7.71 (s,3H, OAc-4"',7); 7.89 (s,3H,OAc-7");

4"',5,5",7,7"-Penta-O-methyl-4'-O-6"-biflavonyl ($AC_{1a}M$) :

AC_{1a} (40 mg) was methylated with methyl iodide (2 ml) and potassium carbonate (2 g) in acetone (150 ml). The methyl ether was purified by preparative thin layer chromatography (silica gel; E.Merck). A white homogeneous product was obtained which crystallised from $CHCl_3$-MeOH as colourless needles (30 mg), m.p. 268-270° R_f 0·52, Mol.wt. 608 (M^+).

NMR (Pyridine) 60 Mc: Values on τ scale :

5.88 (s,3H, OCH_3-5"); 6.12 (s, 3H, OCH_3-5); 6.16 (s, 3H, OCH_3-7"); 6.19 (s, 6H, OCH_3-4"',7).

4',4"',5,5",7-Pentaacetoxy-7"-O-methyl-3',8"-biflavonyl ($AC_{1b}A$):

A mixture of AC_{1b} (60 mg), pyridine (1.5 ml) and acetic anhydride (1.5 ml) was heated on water bath for 2 hrs. After usual work up it crystallized from $CHCl_3$-MeOH (60 mg).

NMR ($CDCl_3$), 100 Mc: Values on τ scale :

1.99 (q,1H, J_1=9 c/s, J_2= 3 c/s,H-6'); 1.99 (d, 1H, J=3 c/s, H-2'); 2.48 (d, 2H, J=9 c/s, H-2"',6"'); 2.53 (d, 1H, J=9 c/s, H-5'); 2.74 (d, 1H, J=3c/s, H-8); 2.95 (d, 1H, J=9 c/s, H-3"',5"'); 3.16 (d, 1H, J=3 c/s, H-6); 3.23 (s, 1H, H-6"); 3.41,3.33 (s,2H, H-3,3"); 6.09 (s,3H,OCH_3-7"); 7.44, 7.50 (s,6H,OAc-5,5"); 7.62, 7.68 (s,6H,OAc-4',4"'); 7.95 (s,3H, OCH_3-7).

4',5,5",7"-Tetrahydroxy-4"',7-di-O-methyl-6,8"-biflavonyl (AC_2):

Crystallized from $CHCl_3$-MeOH as yellow needles (120 mg), m.p. 212-213°, R_f 0.43, λ max (EtOH), 277, 337 nm; (N/500 NaOEt) 287,382 (inflex), 403 nm; Mol.wt. 566 (M^+).

4',5,5",7"-Tetraacetoxy-4"',7-di-O-methyl-6,8"-biflavonyl (AC_2A):

A mixture of AC_2 (60 mg), pyridine (1 ml) and acetic anhydride (1 ml) was heated on a water bath for 2 hrs. It was poured onto crushed ice. The white solid was filtered, washed, dried and crystallized from $CHCl_3$-MeOH as colourless needles (40 mg), m.p. 181-85°, Mol.wt. 734 (M^+).

NMR ($CDCl_3$), 100 Mc: Values on τ scale :

2.08 (d, J=9 c/s, 2H, H-2',6'); 2.73 (d, J=9 c/s, 2H,H-3',5'); 2.60 (d, J=9 c/s, 2H,H-2"',6"'); 3.19 (d, J=9 c/s,2H, H-3"',5"'); 2.99,3.02 (s, 1H each, H-8,6"); 3.38, 3.46 (s, 1H each, H-3,3"); 7.86 (s,3J,OAc-4'); 6.24 (s, 3H, OCH_3-4"'); 6.21 (s, 3H, OCH_3-7); 7.91 (s, 3H, OAc-7"); 7.67 (s, 3H,OAc-5); 7.56 (s, 3H, OAc-5").

4',4"',5,5",7,7"-Hexa-O-methyl-6,8"-biflavonyl (AC_2M) :

AC_2 (60 mg), anhydrous potassium carbonate (2g), methyl iodide (1 ml) and dry acetone (100 ml) were refluxed for 12 hrs. After usual work up the methyl ether was recrystallized from ethanol as colourless needles (40 mg), m.p. 162-64°, Mol.wt.622(M^+).

NMR ($CDCl_3$), 100 Mc: Values on τ scale :

2.12 (d, J=9 c/s,2H,H-2',6'); 2.99 (d, J=9 c/s, 2H,H-3',5'); 2.63 (d, J=9 c/s, 2H,H-2"',6"'); 3.22 (d, J=9 c/s, 2H,H-3"',5"'); 3.09 (s, H,H-8); 3.36 (s,H,H-6"); 3.47, 3.49 (s, 2H,H-3',3"); 6.22, 6.26 (s, 3H each, OCH_3-4',4"'); 6.12, 6.14 (s, 3H each, OCH_3-7,7"); 6.41 (s, 3H, OCH_3-5); 5.95 (s,3H, OCH_3-5").

AC_3 :

AC_3 (92 mg) was subjected to CCD separation (293 transfers) between ethyl methyl ketone and borate buffer (pH 10.00). Two main fractions AC_3X_1 (tubes 66-95, 45 mg) and AC_3X_2 (tubes 106-145, 43 mg) were obtained. Both the fractions were acetylated for NMR studies.

4",5,5",7-Tetraacetyl-4',7"-di-O-methyl-3',8"-biflavonyl (AC_3X_1A) :

A mixture of AC_3X_1 (45 mg), pyridine (1 ml) and acetic anhydride (1 ml) was heated on water bath for 2 hrs. After usual work up it crystallized from chloroform-methanol (25 mg).

NMR ($CDCl_3$), 100 MH_z: Values on τ scale :

2.08-2.1 (q, 1H, J_1= 9 c/s, J_2=3 c/s, H-6'); 2.08-2.1 (d, 1H, J=3 c/s, H-2'); 2.56 (d, 2H, J=9 c/s, H-2"',6"'); 2.87 (d, 1H, J=9 c/s, H-5'); 2.97 (d, 2H, J= 9 c/s, H-3"',5"'); 2.73 (d,1H, J=2 c/s, H-8); 3.25 (s, 1H,H-6"); 3.40, 3.47 (s, 2H,H-3,3"); 6.13 (s,3H,OCH_3-7"); 6.22 (s, 3H,OCH_3-4'); 7.50,7.56 (s, 6H,OAc-5,5"); 7.78 (s, 3H, OAc-7); 7.73 (s,3H, OAc-4"').

4',4"',5,5"-Tetraacetyl-7,7"-di-O-methyl-8,8"-biflavonyl(AC_3X_2A):

A mixture of AC_3X_2 (45 mg), pyridine (1 ml), acetic anhydride (1 ml) was heated on water bath for 2 hrs. After usual work up it crystallised from chloroform-methanol (25 mg), m.p. 288-90°, Mol.wt. 734 (M^+).

NMR ($CDCl_3$), 60 Mc: Values on τ scale :

2.65 (d, 4H, J=9 c/s, H-2',6',2"',6"'); 2.94 (d, 4H, J=9 c/s, H-3',5',3"',5"'); 3.18 (s, 2H, H-6,6"); 3.45 (s, 2H, H-3,3"); 6.08 (s, 6H, OCH_3-7,7"); 7.40 (s, 6H,OAc-5,5"); 7.64 (s, 6H, OAc-4',4"').

AC_4:

AC_4 (120 mg) was subjected to CCD separation between ethyl methyl ketone and borate buffer (pH 9.00). Three fractions, AC_4X_1 (50 mg), AC_4X_2 (20 mg) and AC_4X_3 (15 mg) were recovered.

4"',5,5"-Triacetoxy-4',7,7"-tri-O-methyl-8,8"-biflavonyl (AC_4X_1A):

A mixture of AC_4X_1 (50 mg), pyridine (1 ml) and acetic anhydride (1 ml) was heated on water bath. After usual work up it crystallized from methanol-chloroform (30 mg), m.p. 265°.

NMR ($CDCl_3$), 60 Mc: Values on τ scale :

6.21 (s, 3H,OCH_3-4'); 6.15 (s,6H,OCH_3-7,7"); 7.72 (s,3H, OAc-4"'); 7.47 (s, 6H,OAc-5,5").

5,5",7-Triacetoxy-4',4"',7"-tri-O-methyl-3',8"-biflavonyl (AC_4X_2A):

A mixture of AC_4X_2 (20 mg), pyridine (0.5 ml), acetic anhydride (0.5 ml) were heated on water bath for 2 hrs. The white solid after usual work up crystallized from $CHCl_3$-MeOH (15 mg), m.p.190-91°, Mol. wt. 706 (M^+).

NMR ($CDCl_3$), 60 Mc: Values on τ scale :

6.24 (s, 3H,OCH_3-7"); 6.30,6.42 (s,6H,OCH_3-4',4"'); 7.57 (s, 3H,OAc-5); 7.46 (s, 3H, OAc-5"); 7.79 (s,3H, OAc-7).

5,5",7"-Triacetoxy-4',4"',7-tri-O-methyl-3',8"-biflavonyl (AC_4X_3A) :

A mixture of AC_4X_3(15 mg), pyridine (0.5 ml) and acetic anhydride (0.5 ml) was heated on water bath for 2 hrs. After usual work up it crystallised from $CHCl_3$-MeOH (12 mg); m.p. 266-67°, Mol.wt. 706 (M^+).

NMR ($CDCl_3$), 60 Mc: Values on τ scale :

6.24 (3H,s, OCH_3-4'); 6.43 (3H,s, OCH_3-4"'); 6.28 (3H,s, OCH_3-7); 7.91 (s,3H, OAc-7"); 7.52(s,3H, OAc-5); 7.50 (s,3H, OAc-5").

5,5"-Dihydroxy-4',4"',7,7"-tetra-O-methyl-3',8"-biflavonyl (AC_5) :

Crystallised from $CHCl_3$-MeOH as yellow needles (100 mg) m.p. 276°C, Mol.wt. 594 (M^+).

5,5"-Diacetoxy-4',4"',7,7"-tetra-O-methyl-3',8"-biflavonyl (AC_5A):

A mixture of AC_5 (60 mg), pyridine (1.5 ml) and acetic anhydride (3 ml) were heated at 85-95° on water bath for 2 hrs, cooled and poured over crushed ice. The solid was washed, dried and crystallised from MeOH-EtOAc in the form of colourless needles (50 mg), m.p. 220-24°C, mol.wt. 678 (M^+).

NMR ($CDCl_3$), 100 Mc: Values on τ scale :

2.08 (q, J_1=9 c/s, J_2=3 c/s,H-2'); 2.86 (d,1H, J=9 c/s, H-5'); 2.13 (d, 1H, J=3 c/s, H-6'); 2.64 (d, 2H, J=9 c/s, H-2"',6"'); 3.23 (d, 2H, J=9 c/s, H-3"',5"'); 3.50, 3.44 (s, 2H,H-3,3"); 3.27 (s, 1H,H-6"); 3.42 (d, 1H, J=3 c/s, H-6); 3.19 (d, 1H, J=3 c/s, H-8); 6.16,6.23,6.22 (s, 12H,OMe-4',4"',7,7"); 7.52, 7.59 (s, 6H, OAc-5,5").

5,5"-Dihydroxy-4',4"',7,7"-tetra-O-methyl-8,8"-biflavonyl (AC_6):

The solid crystallized from $CHCl_3$-MeOH as yellow needles (90 mg), m.p. 151°C, Mol.wt. 594 (M^+).

5,5"-Diacetoxy-4',4"',7,7"-tetra-O-methyl-8,8"-biflavonyl (AC_6A):

A mixture of AC_6 (60 mg), pyridine (1.5 ml) and acetic anhydride (3 ml) were heated on water bath for 2 hrs. After usual work up it crystallized from $CHCl_3$-MeOH as colourless needles (45 mg), m.p. 156°C, Mol.wt. 678 (M^+).

NMR ($CDCl_3$), 100 Mc: Values on τ scale :

3.51 (s,2H,H-3,3"); 3.19 (s,2H,H-6,6"); 2.75 (d, 4H, J=9 c/s, H-2',6',2"',6"'); 3.22 (d, 4H, J=9 c/s, H-3',5",3"',5"'); 6.20 (s,6H, OMe-7,7"); 6,26 (s, 6H, OMe-4',4"'); 7.51 (s, 6H, OAc-5,5").

EXTRACTION OF BIFLAVONYLS FROM LEAVES OF CEPHALOTAXUS DRUPACEA Sieb & Zucc :

Air dried and powdered leaves (1.5 kg) were extracted with different solvents as described earlier. The green viscous residue (Ca 3 g) obtained after solvent treatment was subjected to column chromatography (magnesium silicate, Woelm) and eluted with solvents in the increasing order of polarity. The yellow solid mixture of biflavones (2 g) so obtained was separated in four fractions (three major) by preparative thin layer chromatography (silica gel; NCL-Poona). The fractions were labelled as CT_1, CT_2, CT_3 and CT_4.

CT_1 :

Being the minor constituent, CT_1 R_f 0.17, was methylated using methyl iodide and freshly ignited potassium carbonate in dry acetone. The methylated mixture by TLC examination showed the presence of amentoflavone (R_f values and characteristic fluorescence in U.V.light).

4',4"',5,5",7"-Pentahydroxy-7-O-methyl-3',8"-biflavonyl (CT_2) :

Crystallized as light yellow needles (70 mg) from pyridine-MeOH, m.p.300°, R_f 0.37 , λ_{max}^{EtOH} 272 nm ,335nm, Mol.wt. 552 (M^+).

4',4"',5,5",7"-Pentaacetoxy-7-O-methyl-3',8"-biflavonyl (CT_2A) :

CT_2 (35 mg) was acetylated with pyridine (1 ml) and acetic anhydride (1 ml). The acetate crystallized from $CHCl_3$-EtOH as colourless needles (25 mg), m.p.245°, Mol.wt. 762 (M^+). NMR τ ppm: 6.28 (s, 3H,OMe-7); 7.96 (s, 3H,OAc-4'); 7.87 (s, 3H, OAc-4"'); 7.54 (s, 3H,OAc-5); 7.49 (s,3H,OAc-5"); 7.90 (s,3H, OAc-7").

4',4"',5,5",7,7"-Hexa-O-methyl-3',8"-biflavonyl (CT_2M=CTM):

A mixture of CT_1 (35 mg) potassium carbonate (1 g) and methyl iodide (1.5 ml) in dry acetone (150 ml) was refluxed for 10 hrs. The reaction mixture, by usual work up and purification crystallized from $CHCl_3$-MeOH as colourless needles (20 mg) m.p. 225°, R_f 0.40, Mol.wt. 622 (M^+).

NMR ($CDCl_3$), 60 Mc: Values on τ scale :

6.25 (s,6H,OMe 4',7); 6.41 (s,3H,OMe-4"'); 6.13 (s,3H,OMe-5); 5.94 (s,3H,OMe-5"); 6.18 (s,3H,OMe-7").

4"',5,5",7"-Tetrahydroxy-4',7-di-O-methyl-3',8"-biflavonyl (CT_3):

Crystallized as yellow flakes (150 mg) from pyridine-MeOH, m.p. 300°, R_f 0.54; λ_{max}^{EtOH} 275 nm, 335 nm; Mol.wt.566 (M^+).

4"',5,5",7"-Tetraacetoxy-4',7-di-O-methyl-3',8"-biflavonyl(CT_3A) :

A mixture of CT_3 (80 mg) acetic anhydride (1 ml) and pyridine (1 ml) when heated on a water bath for 2 hrs gave an acetate which crystallized from $CHCl_3$-EtOH as colourless needles (60 mg), m.p. 256°, Mol.wt. 734 (M^+).

NMR ($CDCl_3$), 60 Mc: Values on τ scale :

6.27 (s,6H,OMe-4',7); 7.87 (s,3H,OAc-4"'); 7.50 (s,6H,OAc-5,5"); 7.91 (3H,OAc-7").

4',4"',5,5",7,7"-Hexa-O-methyl-3',8"-biflavonyl (CT_3M-CTM) :

On methylation CT_3 gave amentoflavone hexamethyl ether which was characterised by its identical chromatographic and spectral behaviour with CT_2M.

5,5",7"-Trihydroxy-4',4"',7-O-methyl-3',8"-biflavonyl (CT_4) :

Crystallized as yellow needles (160 mg) from pyridine-MeOH; m.p. 295°; R_f 0.61; λ_{max}^{EtOH} 276 nm, 335 nm; Mol.wt. 580 (M^+).

5,5",7"-Triacetoxy-4',4"',7-tri-O-methyl-3',8"-biflavonyl (CT_4A):

Acetylation of CT_4 (80 mg) with acetic anhydride (1 ml) and pyridine (1 ml) yielded a white solid which slowly crystallized from $CHCl_3$-EtOH as colourless needles (60 mg), m.p.266-67°, Mol.wt. 706 (M^+).

NMR τ ppm : 6.24 (s,3H,OMe-4'); 6.43 (s,3H,OMe-4"'); 6.28 (s, 3H,OMe-7); 7.52 (s,3H,OMe-5); 7.50 (s,3H,OAc-5"); 7.91 (s,3H, OAc-7").

4',4"',5,5",7,7"-Hexa-O-methyl-3',8"-biflavonyl (CT_4M-CTM) :

Methylation of CT_4 gave CT_4M which was characterised as amentoflavone hexamethyl ether by its identical chromatographic and spectral behaviour with authentic sample.

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